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13. ABSTRACT *(Maximum 200 Words)*

This is a progress report for the development of tissue engineering strategies for breast reconstruction following tumor resection:

- Task 1, a long-term (12 month) in vivo study using a rat model, was initiated at the end of Year 1 of this grant. The study was completed in this Year 2 cycle of the grant. Biodegradable polymer scaffolds were fabricated, preseeded with preadipocytes (PAs), and implanted subcutaneously for 1-12 months. Quantitative histometric analysis was developed to assess the study.
- The effect of hypoxia on PA viability was completed (in vivo design constraint).
- The mechanisms and adhesion molecules involved in PA-material interactions is 90% complete (important for rational modification of polymer surfaces).
- The development of a large animal model (pig) was initiated to address questions raised in the rat model.
- The development of breast-shaped scaffolds has progressed to a 2nd generation virtual model of the breast based on patient-specific data.
- New polymer hydrogels are being investigated for adipose tissue engineering strategies.
- Support for a PhD and BS candidate as well as two technicians was provided. Academic outcome metrics (publications, presentations, etc) have been positive.

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Table of Contents

INTRODUCTION

The cure for breast cancer is a long-term clinical realization. In the meantime, patients continue to undergo mastectomies as a preventative measure against breast cancer or as a means to surgically resect an existing breast cancer. Conventional procedures for reconstructing breast, or other soft tissue defects requiring adipose tissue, involve "robbing Peter to pay Paul". That is, tissue from a donor site on the patient is used to reconstruct the breast mound. Ideally, the reconstructive goal would be to completely avoid using functional tissues, such as muscle, for soft tissue reconstruction. Considering the fact that the general cost of reconstruction is high, in both the monetary and the physical sense, a need exists to reduce costs and develop innovative reconstruction methodologies. The multidisciplinary efforts of bioengineering and materials science, cell biology, and surgical science can interact through the field of tissue engineering to help produce viable adipose tissue solutions for presently limited reconstructive applications in soft tissue augmentation and, ultimately, for incorporation into compound flap tissue for clinical use to increase soft tissue bulk and help create or repair appropriate superficial body contour and shape where well-vascularized soft tissue is needed. Figure 1 depicts the overall strategy.

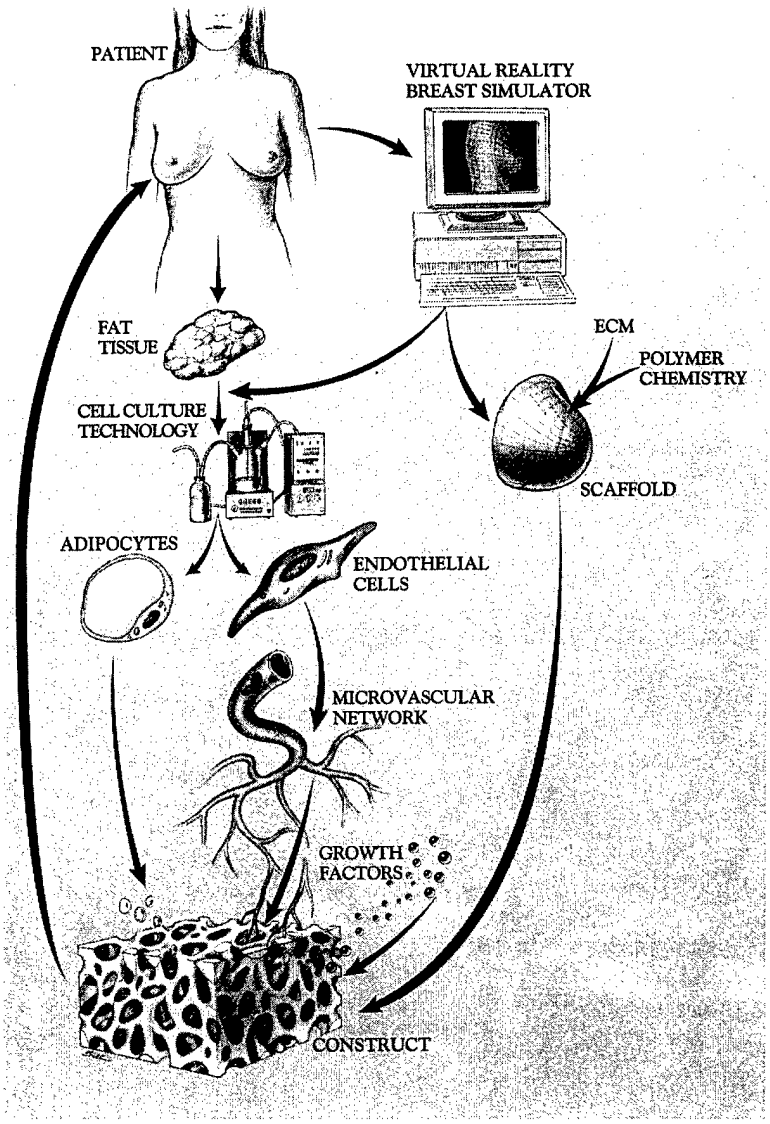


Figure 1. Overall adipose tissue engineering strategy. This grant focuses on the adipose and polymer aspects of the plan. Other grants cover the vascularization issues.

BODY

The specific tasks of the project as originally stated are to:

1. Assess *in vivo* adipose formation in PLGA polymer scaffolds seeded with primary rat preadipocytes and implanted subcutaneously for three and six months. Adipose formation will be assessed histologically using OsO_4 staining.

2. Fabricate breast-shaped PLGA polymer scaffolds using a vacuum-assisted particulate-leaching process and assess polymer architecture. A hemisphere will be used as an initial model breast shape. Pore size distribution, number of pores, and global architecture will be assessed using Hg infusion porosimetry and SEM.

3. Assess feasibility of transferring preadipocyte-seeded, breast-shaped polymer scaffolds as flaps based on an omental vascular pedicle. Conventional tissue transfer and microvascular surgery techniques will be applied to carry out this aim.

Each Task will be addressed separately.

Task 1

Year 1 focused on completing a short-term study[1] (see appendix) and initiating a long-term study. The long-term (12 month) study was completed in Year 2, including all analyses[2] (see appendix). Studies were performed in an effort to develop clinically translatable, tissue engineered adipose constructs for reconstructive, correctional, and cosmetic indications. Rat preadipocytes were harvested, isolated, expanded *ex vivo*, and seeded within PLGA scaffolds, as described in detail in. Preadipocyte-seeded and acellular (control) scaffolds were implanted for times ranging from 1 to 12 months. Explanted scaffolds were stained with osmium tetroxide, processed, and counterstained using H&E. Quantitative histomorphometric analysis was performed on all tissue sections to determine the amount of adipose tissue formed. The analysis protocol was newly developed for this project and consisted of high-resolution digital image acquisition, digital tiling, and multi-band image segmentation, resulting in quantitative classification of adipose tissue and total scaffold area[2]. Analyses revealed maximum adipose formation at 2 months, followed by a decrease at 3 months, and complete absence of adipose and PLGA at 5 through 12 months (Figure 2). These results extend a previous short-term study[1] and demonstrate that adipose tissue can be formed *in vivo* using tissue engineering strategies. However, the long-term maintenance of adipose tissue remains elusive. The inability of the formed fat to persist may be related to the small animal model used, the anatomical location (epididymal fat placed in subcutaneous space), or the fact that the polymer degrades by four months (i.e., fat may need a scaffold to hold it together). These issues will be addressed while conducting Task 3.

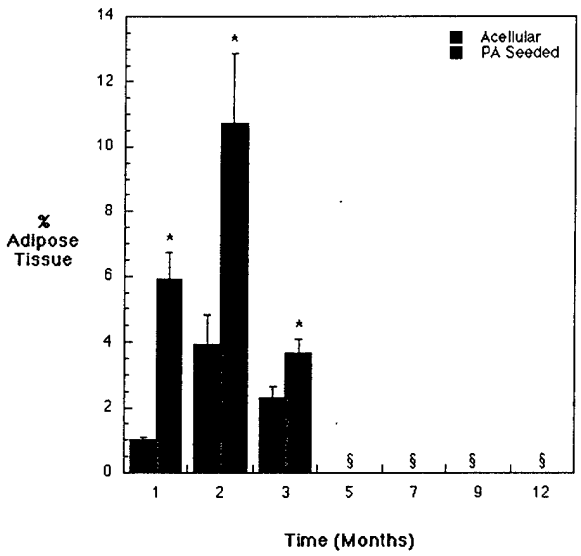


Figure 2. Percent adipose tissue formation in acellular and preadipocyte-seeded scaffolds vs. implantation time. Data are mean±SEM of n=6. * denotes a statistical difference in adipose tissue presence between acellular and PA seeded scaffolds ($p \leq 0.05$). § denotes rats with entirely resorbed PLGA scaffolds and no adipose tissue. Note that acellular constructs do not have 0% adipose tissue because resident preadipocytes from perivascular tissue migrate into the construct.

In addition to the PLGA polymers outlined in the proposal, an industrial research agreement with Johnson & Johnson/Ethicon Endo-Surgery was established to assess several of their proprietary biomaterials. Because of the research agreement between the PI and Johnson & Johnson, material specifications and data cannot be disclosed. Three polymer foams and three non-woven polymer fabrics were assessed by seeding them with preadipocytes and implanting them subcutaneously for 2 months (Figure 3). Adipose tissue formed in all polymers, although none of the polymers exceeded PLGA results published in our short- and long-term studies (data not shown).

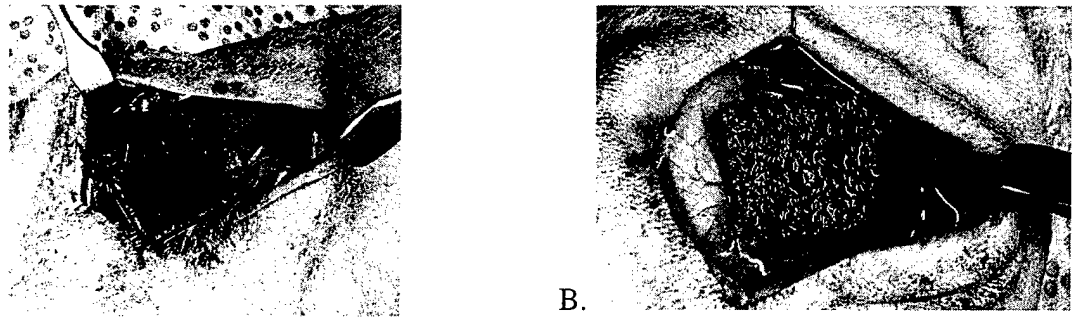


Figure 3. (A) J&J polymer foam implanted subcutaneously. (B) J&J non-woven polymer fabric implanted subcutaneously.

Cells seeded within a tissue construct are initially experience a hypoxic environment. To determine whether preadipocyte or endothelial cell viability are design limitations for in vivo implantation, in vitro hypoxia experiments were conducted. This was initiated in Year 1 and completed in Year 2. Specifically, preadipocyte and microvascular endothelial cell viability were determined under hypoxic conditions utilizing a custom hypoxia chamber and quantitative fluorescence microscopy assay[3]. The hypoxia chamber removed atmospheric and dissolved O_2. Cell apoptosis was qualitatively assessed using a fluorescence and phase microscopy assay

(Figures 4 and 5). Half of the preadipocytes died within 2.3 hours and all cells were dead by 3-3.5 hours when cultured under hypoxic conditions. Marked cell detachment from the culture surface was observed subsequent to preadipocyte death. In contrast to the preadipocytes, microvascular endothelial cell viability remained >70% up to 16 days under hypoxic conditions. Similar to preadipocytes, microvascular endothelial cell detachment from the culture surface was observed subsequent to microvascular endothelial cell death. Preadipocyte and microvascular endothelial cell death occurred via apoptosis. For adipose tissue engineering strategies involving preadipocyte and microvascular endothelial cell transplantation, preadipocytes are the limiting cell type.

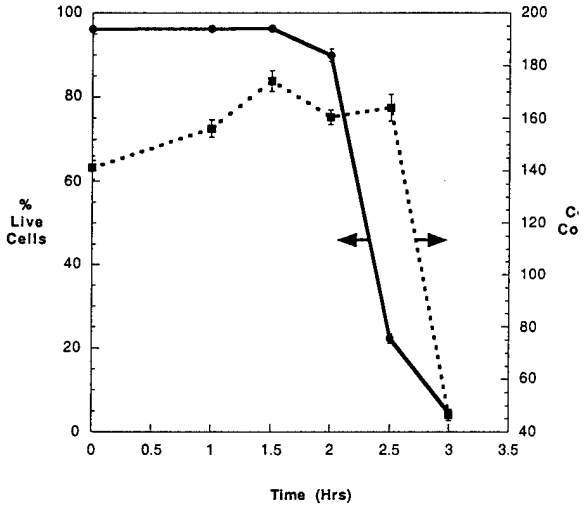


Figure 4. Percent viability (left axis) and total cell number (right axis) vs. time of PAs under hypoxic conditions. Data are mean±SEM (n=5).

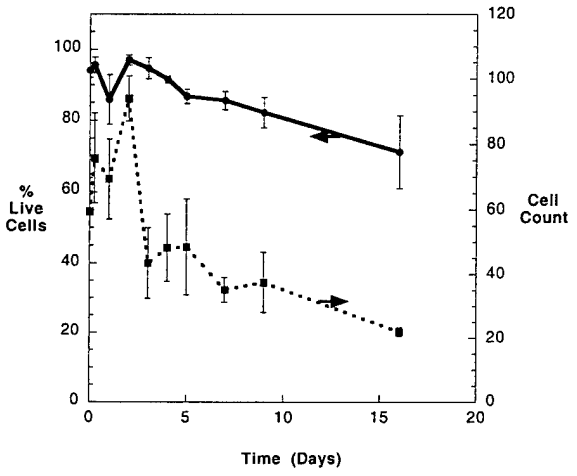
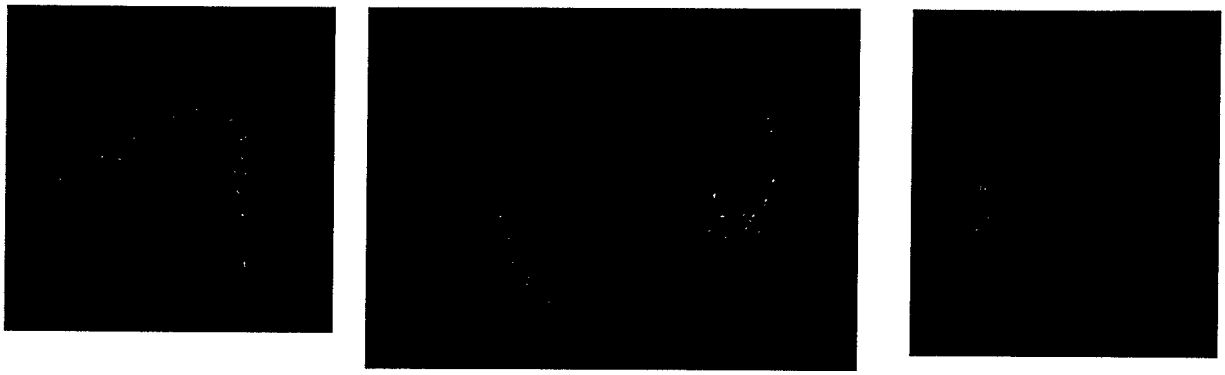


Figure 5. Percent viability (left axis) and total cell number (right axis) vs. time of MECs under hypoxic conditions. Data are mean±SEM (n=4-8).

To develop polymers that posses optimized adhesion characteristics for preadipocytes, the mechanisms involved in their adhesion must be elucidate. This information will be used to derivatize polymer surfaces with adhesion binding sites for preadipocytes. In Year 1, it was determined the preadipocytes prefer binding to laminin over fibronectin, collagen I, or collagen IV using a quantitative cell detachment assay. In Year 2, we have investigated which specific integrin molecules are involved in the binding. This study has involved the use of immunohistochemistry to elucidate integrin subunits expressed by preadipocytes. Integrin

subunits CD29, CD61, and CD49b are expressed on preadipocytes (Figure 6), whereas CD49a, CD49e, or CD49f are not (data not shown). A quantitative cell detachment assay in the presence of monoclonal antibodies to specific integrin subunits (CD61, CD46b, CD29) is currently being used to interogate which of the integrin molecules expressed on preadipocytes are specifically involved with binding to laminin. It should be noted that published data on preadipocyte adhesion is extremely scarce.



CD29 CD61 CD49b

Figure 6. Positive expression of integrin subunits on preadipocytes using fluorescence-conjugated monoclonal antibodies.

Task 2

Our goal is to create a breast-shape polymer scaffold via a hydrogel or laminating a specific polymer foam. We have investigated the use of PLGA[1,2].

In addition, we have investigated other proprietary biomaterials from Johnson & Johnson. Although these polymers show promise and are FDA approved, they are far from ideal for use in breast reconstruction. For instance, the polymer foam scaffolds (like PLGA) are too rigid for placement within the breast envelope. There is concern that the scaffolds would be too uncomfortable to the patient and even wear a hole through the skin envelope. In addition, the polymers we have investigated to date degrade by hydrolysis, which does not degrade at the same rate as tissue formation (i.e., as noted in our long-term PLGA study in Task 1).

To that end, we have initiated collaborative work with Dr. Jennifer West, Rice University to use polymeric hydrogels[4-9]. The hydrogels degrade by proteolysis, this degrading at the same rate as cellular ingrowth. In addition, the hydrogels can be easily derivatized with growth factors (angiogenic and adipogenic) and cell adhesion sequences (e.g. laminin). The first *in vivo* trial is scheduled for the end of August.

In addition, we have initiated a research contract with Fidia Advanced Biopolymers to assess derivatized hyaluronan biomaterials for adipose tissue engineering strategies. They can esterify hyaluronan with adipogenic growth factors and preadipocyte adhesion peptide sequences[10,11].

Task 3

This task was not pursued in Years 1 and pilot studies were performed in Year 2. In Year 3, we plan on completing the development of the large animal model (pig), grow adipose tissue around a vascular pedicle within candidate polymer scaffolds, and then transfer the

adipose/vascular pedicle to a recipient site to assess clinical utility and long-term maintenance of adipose. Preliminary experiments have been conducted and are briefly highlighted. It should be noted that characterized large animal models for adipogenesis do not currently exist.

Initial animal model design has included the development of a subcutaneous defect model. Figure 10 illustrates a subcutaneous adipose tissue defect created and Figure 11 illustrates the result in dimple formation 1 month postop (i.e., impaired contour) resulting from the subcutaneous defects. Adipose strategies will be assessed in part by their ability to restore the adipose tissue deficit.

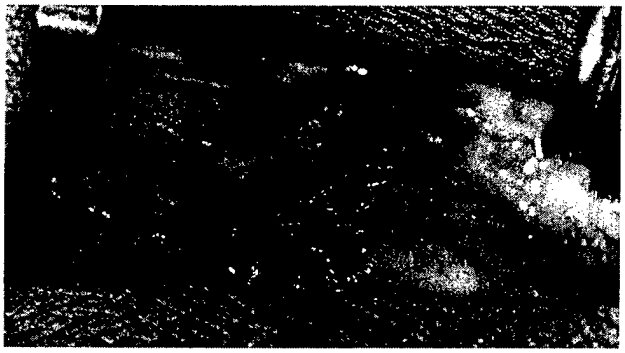


Figure 10. Subcutaneous defect created in a Yucatan micropig. Various size defects were created and assessed.

Figure 11. Impaired contour 1 month postop in a micropig. The tattoos denote the size of the defects originally created subcutaneously.

We have recently hired a new clinical faculty member, Dr. Elizabeth Beahm, who has experience with *de novo* adipose formation around vascular pedicles[12]. This is directly relevant to Task 3 since its goal is to transfer an adipose/vascular pedicle using microsurgery techniques. Previous experiments were conducted in a nude rat model (figures 11 and 12). Matrigel and bFGF were placed around PLA polymer fibers. This construct was then placed around the superficial inferior epigastric vessels of the rat. Constructs in the form of sheets, domes, and spheres were assessed. Results demonstrated fat formation with capillary networks forming off the host vessels. Transference and long-term maintenance could not be assessed since the nude rats do not live greater than 3 months.

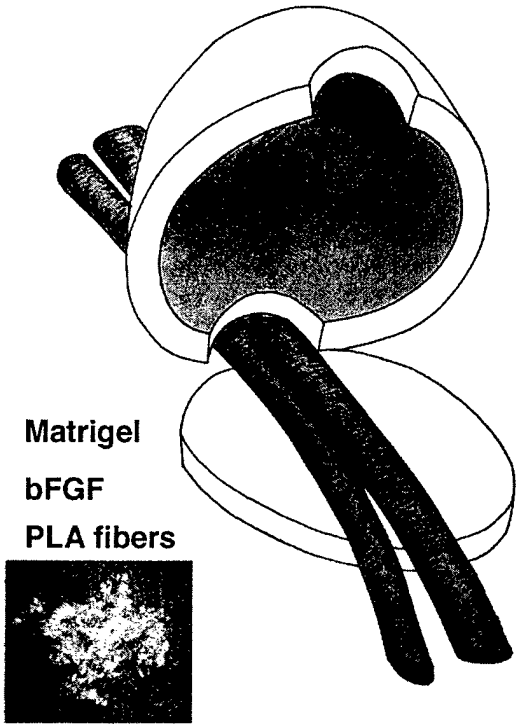


Figure 11. Silicon domes filled with PLA fibers, Matrigel, and bFGF were sutured around an AV loop within a nude rat.

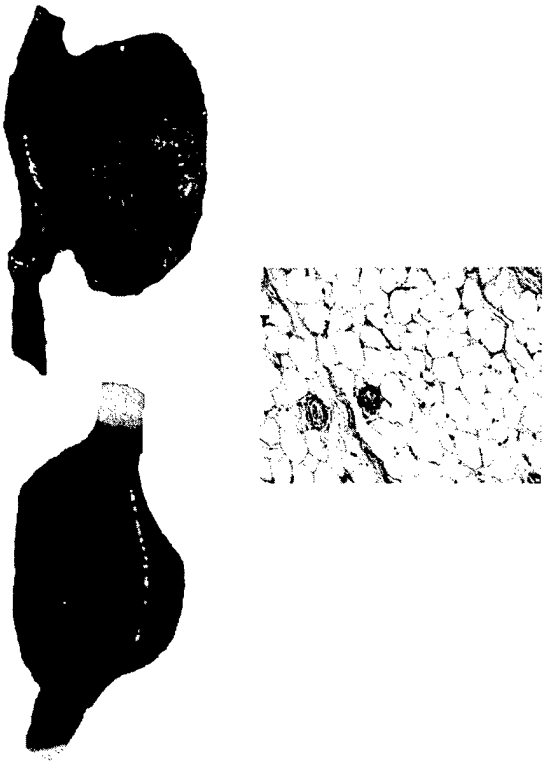


Figure 12. De novo fat tissue formed within the domes. Shown are gross photos of the fat formed as well as a representative histology photo demonstrating well-vascularized fat tissue.

KEY RESEARCH ACCOMPLISHMENTS

- Long-term studies of preadipocyte-seeded PLGA scaffolds demonstrate maximal fat formation at two months. Long-term maintenance of formed fat needs to be addressed in an appropriate animal model.
- Preadipocytes can remain in a hypoxic environment for only ~2 hours before nutrients need to be supplied, by either diffusion or revascularization.
- Various polymer materials have been investigated. Optimal candidates will be assessed in a large animal model.
- Integrin molecules have been shown to be expressed on preadipocyte cell surfaces.
- Two graduate students are working on aspects of this proposal for their PhD thesis.
- Two technicians are working on aspects of this proposal.

- A synergistic clinical faculty member was hired. Army funds were not used, but the presence of this ongoing project aided in hiring the faculty member due to her interest in adipose tissue engineering.

- A large animal model is under development to appropriately address Task 3.

REPORTABLE OUTCOMES

The PI has been promoted to Associate Professor. In addition, he serves as Associate Director of the newly created University of Texas Center for Biomedical Engineering. He continues to hold adjunct positions at three academic institutions. Other academic metrics of importance follow:

(A) Other grant support obtained based in initial Army support:

1. Hydrogel-based adipose tissue engineering, Cancer Fighters of Houston, Principal Investigator, 01/00-12/01, total $20,000.

2. Assessment of preadipocyte-seeded biomaterials for adipose tissue engineering: Short-term study, Johnson & Johnson/Ethicon-EndoSurgery, Principal Investigator, 06/00-06/01, total $20,000.

3. Soft tissue repair using cell-seeded, factor-loaded microparticles, Plastic Surgery Education Foundation, Principal Investigator, 07/99-06/01, total $5,000.

4. A cell-free method to induce adipogenesis for soft tissue augmentation, Plastic Surgery Education Foundation, Co-Investigator (P. Chevray, Principal Investigator), 08/01-07/02, total $5,000.

(B) Manuscripts

1. **Patrick Jr., C.W.**, Chauvin, P.B., Reece, G.P Preadipocyte seeded PLGA scaffolds for adipose tissue engineering. *Tissue Engineering* 5:139-151, 1999.

2. **Patrick Jr., C.W.,** Tissue engineering of fat. *Seminars in Surgical Oncology,* 19:302-311,2000.

3. Brey, E., **Patrick Jr., C.W.** Tissue engineering applied to reconstructive surgery. *IEEE Engineering in Medicine and Biology* 19:122-125, 2000.

4. Miller, M.J., Loftin, B., **Patrick Jr., C.W.**, Rosen, J.M., Siemionow, M., Face to face: A maxillofacial forum: New technologies, *Maxillofacial News* 15(3):5-10, 2000.

5. **Patrick Jr., C.W.,** Emerging technology for the new millennium: Tissue engineering. *Maxillofacial News* 15(3):1-13, 2000.

6. **Patrick Jr., C.W.,** Tissue engineering strategies for soft tissue repair. *Anatomical Record,* (in press).

7. **Patrick Jr., C.W.,** Zheng, B., Johnston, C., Reece, G.P. Long-term implantation of preadipocyte seeded PLGA scaffolds. *Tissue Engineering* (in press).

8. **Patrick Jr., C.W.,** Frye, C., Wu, X., Dempsey, K. Transplantation constraints for adipose tissue engineering. *Tissue Engineering* (submitted).

(C) Abstracts in Peer-Reviewed Journals

1. Kakadiaris, I., Chen, D., Miller, M.J., Loftin, B., and **Patrick Jr., C.W.** Simulation-base determination of breast tissue engineering design parameters. *Tissue Engineering* 6:662, 2000.

2. Wu, X. and **Patrick Jr., C.W.** Comparison of preadipocyte and mature adipocyte hypoxia tolerance. *Tissue Engineering* 6:691, 2000.

3. **Patrick Jr., C.W.,** Reece, G., Johnston, C. Long-term implantation of preadipocyte-seeded PLGA scaffolds. *Annals of Biomedical Engineering* (in press), 2001.

4. **Patrick Jr., C.W.,** Frye, C., Dempsey, K., Wu, X. Transplantation constraints for adipose tissue engineering. *Annals of Biomedical Engineering* (in press), 2001.

5. Sarh, K., Kakadiaris, I.A., Ravi-Chandar, K., Miller, M., Patrick Jr., C.W. Towards a biomechanical model of the breast: A simulation-based study. *Annals of Biomedical Engineering* (in press), 2001.

(D) Book Chapters

1. Chen, D.T., Kakadiaris, I.A., Miller, M.J., Loftin, R.B., **Patrick Jr., C.W.** Modeling for plastic and reconstructive breast surgery. In: Delp, S.L, DiGioia, A.M., Jaramaz, B. (eds) Lecture Notes in Computer Science, pp. 1040-1050, Berlin, Springer, 2000.

2. **Patrick Jr., C.W.,** Wu, X., Johnston, C., Reece, G.P. Epithelial cell culture: Breast. In: Atala, A., Lanza, R. (eds) Methods of Tissue Engineering, pp. 143-154, San Diego, Academic Press, 2001.

3. Robb, G.L., Miller, M.J., **Patrick Jr., C.W.** Breast reconstruction. In: Atala, A., Lanza, R. (eds) Methods of Tissue Engineering, pp. 881-889, San Diego, Academic Press, 2001.

(E) Presentations at National Conferences

1. **Patrick Jr., C.W.,** Elbjerami, W., Chauvin, P.B., Reece, G.P. Adipose tissue engineering. Summer Bioengineering Conference, Big Sky, Montana, 06/99.

2. **Patrick Jr., C.W.,** Tissue engineering, South Texas Society of Plastic Surgery, Austin, Texas, 10/99.

3. Kakadiaris, I., Chen, D., Miller, M.J., Loftin, B., and **Patrick Jr., C.W.** Simulation-based determination of breast tissue engineering design parameters. Tissue Engineering Society, Orlando, Florida, 11/00-12/00.

4. Roweton, S., Freeman, L., **Patrick Jr., C.W.,** Dempsey K., Zimmerman, M. Preadipocyte-seeded absorbable matrices. Johnson & Johnson Excellence in Science Symposium, New Jersey, 11/00.

5. Wu, X. and **Patrick Jr., C.W.** Comparison of preadipocyte and mature adipocyte hypoxia tolerance. Tissue Engineering Society, Orlando, Florida, 11/00-12/00.

6. Parker, T., Miller, M.J., **Patrick Jr., C.W.,** King, T.W., Ames, F.C., Robb, G.L. Aesthetic refinements in autologous breast reconstruction: Tailored breast skin envelope by the wise pattern. American Society for Reconstructive Microsurgery, San Diego, California, 01/01.

7. **Patrick Jr., C.W.,** Reece, G., Johnston, C. Long-term implantation of preadipocyte-seeded PLGA scaffolds. Biomedical Engineering Society, Durham, North Carolina, 10/01.

8. **Patrick Jr., C.W.,** Frye, C., Dempsey, K., Wu, X. Transplantation constraints for adipose tissue engineering. Biomedical Engineering Society, Durham, North Carolina, 10/01.

9. Sarh, K., Kakadiaris, I.A., Ravi-Chandar, K., Miller, M., **Patrick Jr., C.W.** Towards a biomechanical model of the breast: A simulation-based study. Biomedical Engineering Society, Durham, North Carolina, 10/01.

10. Sarh, K., Kakadiaris, I.A., Ravi-Chandar, K., Miller, M., **Patrick Jr., C.W.** Towards a biomechanical model of the breast: A simulation-based study. Advances in Bioengineering, American Society of Mechanical Engineering, New York, New York, 11/01.

(E) Presentations at Universities/Companies

1. **Patrick Jr., C.W.,** Robb, G.L, Miller, M.J. Hydrogel-based adipose tissue engineering, Cancer Fighters of Houston Committee, The University of Texas M.D. Anderson Cancer Center, Houston, Texas, 10/99.

2. **Patrick Jr., C.W.** Tissue engineering indications for reconstructive surgery at UTMDACC, Board of Visitors, The University of Texas M.D. Anderson Cancer Center, Houston, Texas, 11/99.

3. **Patrick Jr., C.W.** Tissue engineering applied to reconstructive surgery, Johnson & Johnson Corporate Biomaterials Center, Somerville, New Jersey, 11/99.

4. **Patrick Jr., C.W.**, Morrison, S., Robb, G.L. Plastic Surgery Program, Presented to UTMDACC's Clinical Research Council, The University of Texas M.D. Anderson Cancer Center, Houston, Texas, 03/00.

5. **Patrick Jr., C.W.** Boob in a box? Rationale and state-of-the-art of breast tissue engineering, Integrative Graduate Education and Research Training lecture, The University of Texas at Austin, Austin, Texas, 9/00.

6. **Patrick Jr., C.W.** Engineering approach to breast reconstruction, University of Maryland Baltimore County, Baltimore, Maryland, 10/00.

7. **Patrick Jr., C.W.** Advances in reconstructive breast surgery, South Texas Society of Plastic Surgery, Irving, Texas, 11/00.

8. Wu, X., Dempsey, K., and **Patrick Jr., C.W.** Preadipocyte cell adhesion to ECM proteins optimized for adipose tissue engineering, 1st Annual Biomedical Engineering Center & Department Retreat, The University of Texas at Austin, Austin, Texas, 12/00.

9. **Patrick Jr., C.W.** Advances in reconstructive surgery, Chancellor's Council, The University of Texas M.D. Anderson Cancer Center, Houston, Texas, 2/01.

10. **Patrick Jr., C.W.** UT Center for Biomedical Engineering: Tissue Engineering, Advances in Oncology Institutional Grand Rounds, The University of Texas M.D. Anderson Cancer Center, Houston, TX, 03/01.

11. **Patrick Jr., C.W.** Plastic surgery and tissue engineering, 1st Annual Biomedical Engineering Center & Department Retreat, The University of Texas at Austin, Austin, Texas, 12/00.

CONCLUSIONS

Task 1 is complete. We have demonstrated that preadipocytes can be seeded within PLGA scaffolds and that fat tissue forms and is well vascularized. The fat formation is maximal at 2 months and begins to resorb at 3 months. Continued efforts need to elucidate strategies to prevent adipose resorption so that long-term persistence is realized. The inability of the formed fat to persist may be due to the small animal model or polymer utilized. Future studies in a large animal model will address these concerns.

Task 2 is progressing nicely, although slight changes have been made in the specific aims. We have begun investigating the use of additional polymers (e.g. hydrogels, hyaluronan) that may be better candidates than PLGA.

Task 3 will begin this year. Pilot studies are complete. A new clinical faculty has been hired with experience in creating adipose tissue around a vascular pedicle within a small animal model. We will combine the vascular pedicle model and our adipose construct model within micropigs to address the long-term maintenance and transference of fat.

REFERENCES:

1. Patrick Jr., C.W., Chauvin, P.B., Reece, G.P. Preadipocyte seeded PLGA scaffolds for adipose tissue engineering. Tissue Eng **5**, 139-151, 1999.

2. Patrick Jr., C.W., Zheng, B., Johnston, C., Reece, G.P. Long-term implantation of preadipocyte seeded PLGA scaffolds. Tissue Engineering in press.

3. Patrick Jr., C.W., Frye, C., Wu, X., Dempsey, K. Transplantation constraints for adipose tissue engineering. Tissue Engineering submitted.

4. Bohl, K.S., West, J.L. Nitric oxide-generating polymers reduce platelet adhesion and smooth muscle cell proliferation. Biomaterials **21**, 2273-2278, 2000.

5. Hill-West, J.L., Chowdhury, S.M., Spleian, M.J., Hubbell, J.A. Inhibition of thrombosis and intimal thickening by *in situ* photopolymerization of thin hydrogel barriers. Proc Nat Acad Sci, USA **91**, 5967-5971, 1994.

6. Mann, B.K., Tsai, A.T., Scott-Burden, T., West, J.L. Modification of surfaces with cell adhesion peptides alters extracellular matrix deposition. Biomaterials **20**, 2281-2286, 1999.

7. Mann, B.K., Schmedlen, R.H., West, J.L. Tethered-TGF-B increases extracellular matrix production of vascular smooth muscle cells. Biomaterials **22**, 439-444, 2001.

8. Sershen, S.R., Westcott, S.L., Halas, N.J., West, J.L. Temperature-sensitive polymer-nanoshell composites for photothermaly modulated drug delivery. J Biomed Mater Res **51**, 293-298, 2000.

9. West, J.L., Hubbell, J.A. Separation of the arterial wall from blood contact using hydrogel barriers reduces intimal thickening after balloon injury in the rat: The roles of medial and luminal fators in arterial healing. Proc Natl Acad Sci **93**, 13188-13193, 1996.

10. Campoccia, D., Doherty, P., Radice, M., Brun, P., Abatangelo, G., Williams, D.F. Semisynthetic resorbale materials from hyaluron esterification. Biomaterials **19**, 2101-2127, 1998.

11. Abatangelo, G., Weigel, P.H. Redefining Hyaluronan. Amstedam: Elsevier; 2000.

12. Beahm, E., Wu, L., Walton, R.L. Lipogenesis in a vascularized engineered construct. American Society of Reconstructive Microsurgeons. San Diego, CA; 2000

APPENDICES

1. Manuscripts

IEEE Engineering in Medicine and Biology 19:122-125, 2000
Seminars in Surgical Oncology, 19:302-311,2000.
Tissue Engineering 5:139-151, 1999
Tissue Engineering . in press, 2001

2. Book Chapters

Epithelial cell culture: Breast. In: Atala, A., Lanza, R. (eds) Methods of Tissue Engineering, pp. 143-154, San Diego, Academic Press, 2001.

Breast Reconstruction. In: Atala, A., Lanza, R. (eds) Methods of Tissue Engineering, pp. 881-889, San Diego, Academic Press, 2001.

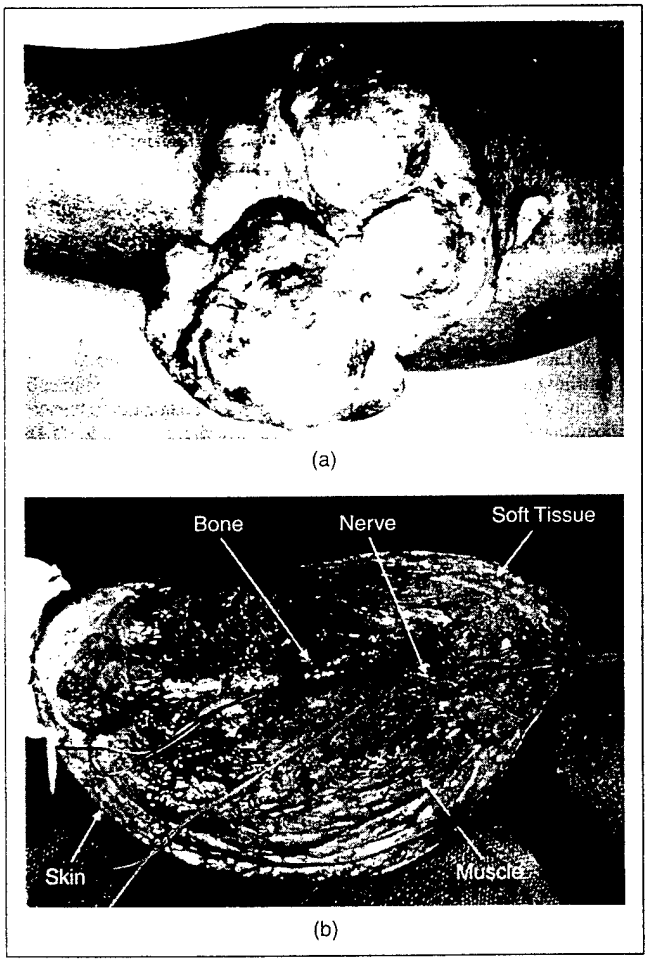
Cellular/Tissue Engineering

Maria Papadaki

Tissue Engineering Applied to Reconstructive Surgery

Eric M. Brey and Charles W. Patrick Jr.

Tissue engineering has excited surgeons and scientists alike due to its potential to revolutionize plastic and reconstructive surgery [1-8]. In fact, applications in reconstructive surgery were heralded as future challenges for tissue engineering at the Tissue Engineering Society's inaugural meeting [2]. Plastic surgery is an art and science that attempts to improve the quality and length of a patient's life through the replacement of the functional and aesthetic qualities of lost or removed tissue [6]. The impetus for tissue engineering parallels these goals. Tissue engineering attempts to develop "biological substitutes that restore, maintain, or improve tissue function" [3].



1. Example of tissue defects clinicians must repair. (a) Patient with tumor growth on lower leg. The tumor is extirpated (removed) by oncological surgeons. (b) The resulting tumor defect must be repaired by reconstructive surgeons. The defect involves skin, soft tissue, nerve, bone, and muscle. The extent of the defect precludes primary closure and must currently be reconstructed using a free flap harvested from a donor site on the patient.

Currently, the gold standard for tissue reconstruction involves the transfer of uninjured tissue, either with (flaps) or without (grafts) blood supply, to repair a defect [6, 7] (Fig. 1). Although there have been great strides in tissue transfer techniques, methods of modifying tissue have advanced little in the past four centuries [7]. Also, by relying on donor tissue, surgeons are limited by tissue availability, associated donor site morbidity, and prolonged hospital time. Tissue engineering offers a strategy to preclude the use of donor tissue.

This review provides examples of tissue engineering applied to reconstructive surgery. Due to the breadth of the subject matter and page limitations, this review is illustrative rather than comprehensive. This naturally leads to excessive reference to the authors' and colleagues' own work, for which we apologize. The reader is referred to more extensive reviews and primary manuscripts when appropriate. To this end, this review will concentrate on the tissue engineering research currently active within the University of Texas M.D. Anderson Cancer Center's Plastic Surgery Department under the auspices of the Laboratory of Reparative Biology and Bioengineering.

Clinicians, scientists, and engineers are working together to develop techniques for combining the surgical, biological, and engineering sciences in the form of tissue engineering to bring tissue engineering theory into practice. With the broad potential of tissue engineering, we must focus on areas vital to clinical application. Our research is being conducted in four research areas; namely adipose, bone, peripheral nerve, and microvascular tissue engineering.

Microvascular Tissue Engineering

One of the primary limitations in the development of clinically translatable tissue-engineered products is establishing a viable microvascular network [5, 9, 10]. Engineered skin (epidermis) has been successful because the tissue can survive by nutrient diffusion alone, and cartilage is not limited because it is relatively avascular, having low metabolic requirements. All other tissue require a patent microvascular system. The microvascular network limitation of tissue engineering was realized as early as 1973 [11]. Investigators are developing methods to study angiogenesis within natural and synthetic scaffolds on a cellular and molecular level. This knowledge can be used to develop techniques to promote vascular formation in engineered tissue. Although the mechanisms of microvascular formation in physiologic and pathologic (e.g., tumor) situations are being studied extensively, little is known about capillary network formation in engineered tissue [9,12,13,14].

At the Laboratory of Reparative Biology and Bioengineering, we are approaching the issue of microvascular formation from

many fronts. First, the biology and geometry of angiogenesis is being explored to elucidate design parameters for engineering properly vascularized tissue. A combination of a parallel plate flow chamber, bioreactor, and gene chips is used to study the effects of microvascular shear and physiological conditions on microvascular endothelial cells. Hemodynamic forces are theorized to influence neovascularization [15-18] through regulation of cellular molecules associated with endothelial cell proliferation and vessel inosculation. Vascular microarchitecture in natural and synthetic scaffolds can be evaluated with model tissue engineered constructs used in unison with a novel semi-automated method for three-dimensional visualization of tissue samples [19].

Concomitantly, methods for modulating vascularization and vessel inosculation are being studied. To obtain a patent vascular system, an engineered vascular network must inosculate, or fuse, with an existing network to establish a blood supply. Inosculation is one of the primary biological mechanisms that currently allows clinicians to use grafts and flaps to repair tissue defects. Little is known about the underlying biological and molecular mechanisms of vessel inosculation. The contribution of the endothelial cell cell-cell adhesion molecule CD144 is being investigated as an initial attempt to elucidate the mechanism. To determine methods for modulating vascularization, model tissue-engineered constructs are seeded with biodegradable poly(DL-lactic-co-glycolic acid) (PLGA) microspheres [20] containing vascular endothelial growth factor (VEGF) and other growth factors. The timed release kinetics of these microspheres allows for modulation of angiogenesis on a temporal level.

Knowledge acquired about biology, geometry, and the modulation cascade of capillary formation will be put to use through a collaboration with Microfab Technologies, Inc. A bioprinter that can simultaneously print polymers, live cells, and biological macromolecules from multifluid print heads is under development. This printer will allow for the fabrication of "designer tissue" containing patent vascular constructs.

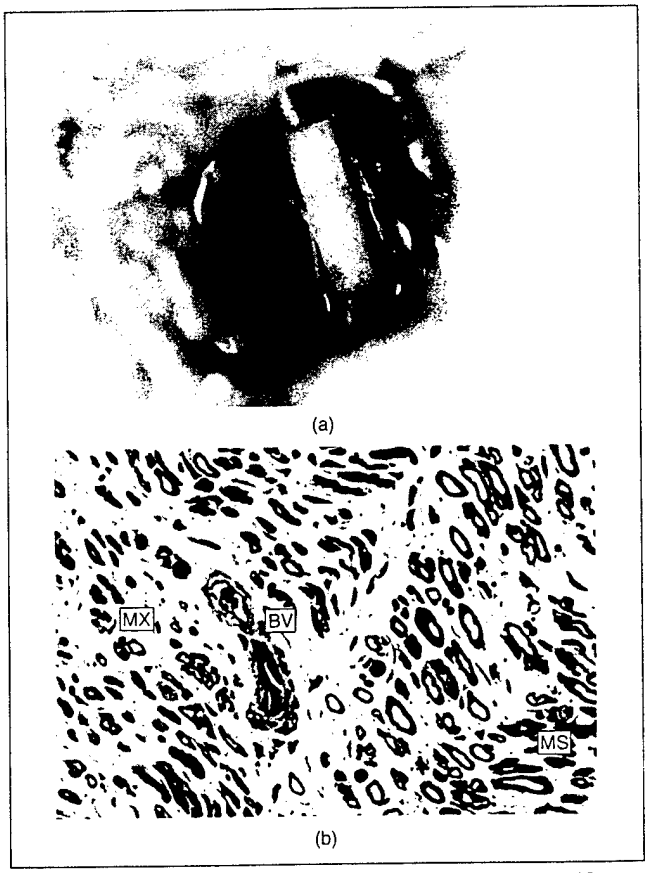
Adipose Tissue Engineering

The prevalence of breast cancer and the need for reconstruction following mastectomy indicate a potential application for engineered tissue. By developing a biocompatible scaffold seeded with autologous cells, a tissue-engineered product could alleviate immune response issues associated with silicone implants and donor site morbidity relating to free flap transfer. The free transfer of adipose tissue is typically unsuccessful due to significant but inconsistent loss of graft volume that forces a substantial amount of "guess work" by the surgeon [21].

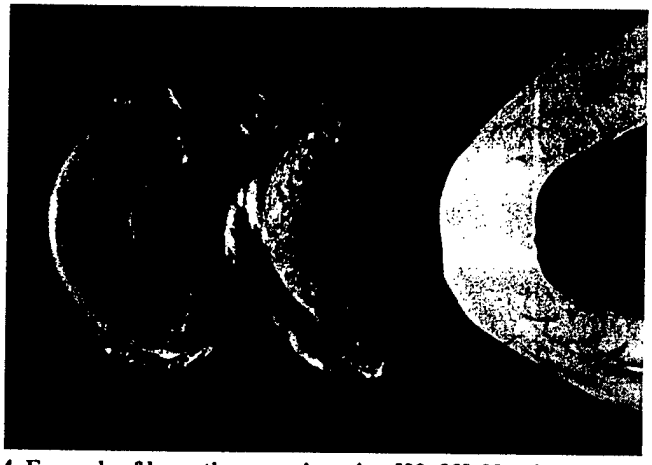
The optimum conditions for engineering adipose tissue are being determined by combining cell culture technology, polymer chemistry, and cell biology. Mature adipocytes cannot be used as a cell source for tissue engineering applications because they are too fragile and do not proliferate [21]. Preadipocytes, in contrast, readily proliferate, are mechanically stable, and withstand hypoxic conditions. We have shown the potential of using primary preadipocytes seeded on PLGA scaffolds in adipose engineering [22] (Fig. 2). Building on these initial results, we are exploring the long-term ability of preadipocytes seeded on PLGA to promote formation of replacement adipose tissue. Others have used a tissue engineering approach for nipple reconstruction of the breast [23]. Techniques for the replacement of small-volume fat defects using other scaffolds are being explored, in particular using hydrogels seeded with preadipocytes.

Working with researchers at the Virtual Environments Research Laboratory, a joint program between the University of Houston and NASA/Johnson Space Center, we are developing a

2. Example of an adipose tissue engineering application for the fabrication of adipose constructs used to restore soft tissue defects [22]. Scanning electron micrograph (SEM) of a preadipocyte-seeded PLGA scaffold. The preadipocytes have been proliferated and differentiated to fill the pores of a PLGA scaffold with mature adipocytes. Bar denotes 300 μm.



3. Example of peripheral nerve tissue engineering. (a) A 12 mm nerve conduit composed of PLLA/PEG has been placed between a transected rat sciatic nerve to serve as a regenerative scaffold [25, 30]. (b) TEM of peripheral nerve tissue that has grown within the conduit (cross section of mid-conduit). Myelin sheaths (MS), matrix (MX), blood vessels (BV), and axons are denoted (images courtesy of G.R.D. Evans, M.D., Univ. of Texas M.D. Anderson Cancer Center).



4. Example of bone tissue engineering [32, 33]. New bone has been formed in the shape of a human mandible within a tissue fabrication system that was placed against the periosteum (a highly vascular layer of tissue that covers the surface of bone and has good bone-producing properties) of an ovine rib (image courtesy of M.J. Miller, M.D., Univ. of Texas M.D. Anderson Cancer Center).

virtual-reality simulation that will allow a surgeon to plan and rehearse a reconstructive breast surgery based on patient-specific data. Not only will this assist in clinical training and surgical planning, but developing the algorithms describing the geometry and heterogeneous properties of adipose tissue will help determine design parameters critical for the development of large-volume adipose tissue engineering for breast reconstruction. As our knowledge of scaffold properties increases, the model will allow for simulation of different tissue engineering techniques within the "virtual patient."

Peripheral Nerve Regeneration

The loss of critical nerves is often realized in tumor extirpation, trauma, or congenital abnormalities [24, 25], and nerve transfer can result in sensory loss at the donor site [5]. Research first needs to establish a proper scaffold for nerve regeneration. Using a salt-leaching and polymer extrusion technique, hollow conduits for replacement of nerve defects have been fabricated. Sutured to existing nerve ends to "guide" tissue regeneration [25, 26] (Fig. 3), the porous PLGA or poly(L-lactic acid) (PLLA) tubes are designed with pore sizes large enough to allow for nutrient diffusion to the lumen but small enough to restrict invasion of scar tissue [26]. The small pore size also keeps cells and associated molecules that enter the lumen from the nerve ends from exiting to surrounding tissue [27]. Nerve growth proceeds from the proximal end through the lumen of the conduit, which must be large enough to prevent compression of the regenerating nerve [28] yet small enough to support it [29]. We have demonstrated improved performance of PLLA over PLGA for nerve regeneration [25].

Support cells and growth factors have to be incorporated into the scaffold to enhance bioactivity. We observed unsatisfactory nerve regeneration when Schwann cells were seeded within the conduits [30]. We have since genetically modified dermal fibroblasts to act like Schwann cells by secreting nerve growth factor (NGF) [31]. The transfected cells can be prompted to increase or inhibit growth factor production depending on various signaling molecules.

In addition to studies involving conduits, in collaboration with LifeCell Corporation, we are assessing the functional outcomes of a proprietary acellular nerve product. The nerve product consists of decellularized nerve tissue from cadavers. The decellularized product does not possess any immunogenic MHCs but maintains the structure and types of extracellular matrices found in peripheral nerve tissue.

Bone Tissue Engineering

Mandible reconstruction due to tumor ablation is a difficult process due, in part, to the complex three-dimensional geometry of the bone [32]. Tissue engineering can offer better results by the ability to customize patient-specific geometry along with the aforementioned improvements in donor site morbidity.

In order to evaluate tissue engineering modalities and bone substitute products, we have developed a bone regeneration model in sheep [32, 33]. Biocompatible chambers restricting bone growth to one dimension containing various scaffolds or bone substitutes are sutured to the periosteum, utilizing its osteogenic potential and high vascularity for improved formation of well vascularized bone. Morcellized bone has been used as a positive control to evaluate tissue engineering scaffolds and bone substitutes [32-34] (Fig. 4). Morcellized corticancellous bone contains the components (various cell types, matrix proteins, growth factor cocktail) of an ideal graft in concentrations optimal for formation of normal bone.

This bone regeneration model is in use to evaluate the ability of our osseous engineering techniques to promote increased development of complex three-dimensional vascularized bone grafts. By varying the chamber shape, scaffold within, and implanted molecules, we can evaluate our ability to form strong, well-vascularized bone in complex three-dimensional shapes.

Conclusion

Tissue engineering has the potential to change the way reconstructive surgery is practiced as well as increase patient quality of life. This review has illustrated a small fraction of the areas in reconstructive surgery that can benefit from tissue engineering. We at the University of Texas M.D. Anderson Cancer Center believe that the best way to bring this potential to fruition is by bringing together experts from the operating room, engineering, academia, and industry, and combining the abilities and knowledge of each to obtain results that could not be accomplished independently.

Acknowledgments

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Charles W. Patrick Jr. received the B.S.Ch.E. degree in chemical engineering with a minor in chemistry from Louisiana State University in 1990 and the Ph.D. degree in chemical engineering from Rice University in 1994. From 1994 to 1996 he was a postdoctoral fellow at the Institute of Biosciences and Bioengineering, Rice University. He became assistant professor of plastic surgery at the University of Texas M.D. Anderson Cancer Center in 1996. He also holds the position of Director, Laboratory of Reparative Biology and Bioengineering at the University of Texas M.D. Anderson Cancer Center. In addition, he is currently an adjunct faculty member of both the Department of Bioengineering, Rice University, and the Graduate School of Biomedical Sciences, University of Texas. His interests involve the application of tissue engineering to reconstructive surgery in the areas of adipose, bone, peripheral nerve, and microvasculature.

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References

1. **Habal MB:** Perspectives on what's new in plastic surgery: The new millennium. *Clin Plast Surg* 23: 1-2, 1996.
2. **Hellman KB:** Directions in tissue engineering: Technologies, applications, and regulatory issues. *Inaugural Meeting of the Tissue Engineering Society*. Orlando, FL, 13-15 December, 1996.
3. **Langer R and Vacanti CA:** Tissue engineering. *Science* 260: 920-926, 1993.
4. **Patrick CW Jr, Mikos AG, and McIntire LV:** Prospectus of tissue engineering. In: Patrick CW Jr. Mikos AG. McIntire LV (Eds): *Frontiers in Tissue Engineering*. New York: Elsevier Science Ltd, pp. 213-232, 1998.
5. **Patrick CW Jr and Evans GRD:** Tissue engineering applied to cancer plastic and reconstructive surgery. In: Ikada Y, Yamaoka Y (Eds): *Tissue Engineering for Therapeutic Use 1*. New York: Elsevier Science Ltd, pp. 97-110, 1998.
6. **Miller MJ and Evans GRD:** Tissue engineering and plastic surgery. In: Patrick CW Jr, Mikos AG, McIntire LV (Eds): *Frontiers in Tissue Engineering*. New York: Elsevier Science Ltd, pp. 213-232, 1998.
7. **Miller MJ:** *Biomaterials: New Challenges and Opportunities*. New York: American Institute of Chemical Engineers, pp. 1-3, 1999.
8. **Patrick CW, Jr:** Emerging technology for the new millenium: Tissue engineering. *Maxillofacial Newsletter*. 15: 1-13, 2000.
9. **Black AF, Berthod F, L'Heurex N, Germain L, and Auger FA:** *In vitro* reconstruction of a human capillary-like network in a tissue engineered skin equivalent. *FASEB J* 12: 1331-1340, 1998.
10. **Reece GP and Patrick CW, Jr:** Tissue engineering construct design principles. In: Patrick CW Jr, Mikos AG, McIntire LV (Eds): *Frontiers in Tissue Engineering*. New York: Elsevier Science Ltd, pp. 167-196, 1998.
11. **Folkman J and Hochberg M:** Self regulation of growth in three dimensions. *J Experiment Med*138: 745-753, 1973.
12. **Holder WD, Jr, Gruber HE, Roland WD, Moore AL, and Culberson CR, et al.:** Increased vascularization and heterogeneity of vascular structures occurring in polyglycolide matrices containing aortic endothelial cells implanted in the rat. *Tissue Engineering* 3: 149-160, 1997.
13. **Knighton DR:** Regulation of wound healing angiogenesis: effect of oxygen gradients in inspired oxygen concentration. *Surgery* 90: 262, 1981.
14. **Soejima K and Negishi N et al.:** Effect of cultured endothelial cells on angiogenesis in vivo. *Plast Reconstr Surg* 101: 1552-1560, 1998.
15. **Hudlicka O:** Growth of vessels-historical review. In: Hammerson F, Hudlicka O (Eds): *Progress in Applied Microcirculation*. Vol. 4, Basal, Karger, pp. 1-8, 1984.
16. **Patrick CW. Jr and McIntire LV:** Shear stress and cyclic strain modulation of gene expression in endothelial cells. *Blood Purif* 13: 112-124, 1995.
17. **Patrick CW Jr, Sampath R, and McIntire LV:** Fluid shear stress effects on cellular function. In: Bronzino (Ed): *Biomedical Engineering Handbook*. Boca Raton FL: CRC Press, pp. 1626-1645, 1995.
18. **Patrick CW Jr and McIntire LV:** Bioengineering contributions in vascular biology at the cellular and molecular level. *Trends Cardiovasc Med* 6: 122-129, 1996.
19. **King TW and Patrick CW Jr:** From plant roots to capillaries: Three dimensional reconstruction of microvascular networks as a model for tissue engineering design. Keystone Tissue Engineering Conference, Copper Mountain, Colorado, January 1998.
20. **King TW and Patrick CW Jr:** Development and *in vitro* characterization of vascular endothelial growth factor (VEGF)-loaded poly(DL-lactic-co-glycolic acid)/poly(ethylene glycol) microspheres using a solid encapsulation/single emulsion/solvent extraction technique. *J Biomed Mater Res* 51: 383-390, 2000..
21. **Patrick CW Jr, Chauvin PB, and Robb GL:** Adipose tissue engineering. In: Patrick C, Jr, Mikos AG, McIntire LV (Eds): *Frontiers in Tissue Engineering*. New York: Elsevier Science Ltd, pp. 369-382, 1998.
22. **Patrick CW, Jr, Chauvin PB, Hobley J, and Reece GP:** Preadipocyte seeded PLGA scaffolds for adipose tissue engineering. *Tissue Engineering* 5: 139-151, 1999.
23. **Cao YL, Lach E, Kim TH, Rodriguez A, Arevalo CA, and Vacanti CA:** Tissue-engineered nipple reconstruction. *Plast Reconstr Surg* 102: 2293-2298, 1998.
24. **Brandt K, Evans GRD, Gurlek A, Savel T, Lohman R, et al.:** The effects of preoperative irradiation on peripheral nerve regeneration. *Ann Plast Surg* 40: 277-282, 1998.
25. **Evans GRD, Brandt K, Widmer MS, Lu L, Meszlenyi RK, et al.:** In vivo evaluation of poly(L-lactic acid) porous conduits for peripheral nerve regeneration. *Biomaterials* 20: 1109-1115, 1999.
26. **Widmer MS, Gupta PK, Lu L, Meszlenyi RK, Evans GRD, et al.:** Manufacture of porous biodegradable polymer conduits by an extrusion process for guided tissue regeneration. *Biomaterials* 19: 1945-1955, 1998.
27. **Jenq C and Coggeshall E:** Nerve regeneration through holey silicone tubes. *Brain Res* 361: 233-241, 1985.
28. **Mackinnon SE, Dellon AL, Hudson AR, Hunter DA:** Chronic nerve compression - An experimental model in the rat. *Ann Plast Surg* 13: 112-120, 1984.
29. **Ducker TB and Hayes GJ:** Experimental improvements in the use of silastic cuff for peripheral nerve repair. *J Neurosurg* 28: 582-587, 1968.
30. **Evans GRD, Brandt K, Katz S, Chauvin P, Otto L, et al.:** Bioactive poly(L-lactic acid) conduits seeded with Schwann cells for peripheral never regeneration. *Plast Reconstr Surg*, to be published.
31. **Patrick CW, Jr, Zheng B, Schmidt M, Herman S, Chauvin P, et al.:** Dermal fibroblasts genetically engineered to release NGF for peripheral nerve regeneration. *Exp Neurol*, to be published.
32. **Miller MJ, Goldberg DP, Yasko AW, Lemon JC, Satterfield WC, et al.:** Guided bone growth in sheep: a model for tissue-engineered bone flaps. *Tissue Engineering* 2: 51-59, 1996.
33. **Thomson RC, Mikos AG, Beahm E, Lemon JC, Satterfield WC, et al.:** Guided tissue fabrication from periosteum using preformed biodegradable polymer scaffolds. *Biomaterials* 20: 2007-2018, 1999.
34. **Patrick CW, Friedrich J, and Miller MJ:** Computer-assisted histometric analysis of tissue-engineered ovine bone. *Analytical and Quantitative Cytology and Histology* 20: 199-206, 1998.

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Adipose Tissue Engineering: The Future of Breast and Soft Tissue Reconstruction Following Tumor Resection

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Reconstructive surgeons have always been at the forefront of medical technology. The history of reconstructive surgery began with ablative surgery, which was followed by tissue and organ transplantation, leading to contemporary tissue reconstruction. The field of reconstructive surgery is poised at the next stage of its evolution, namely tissue regeneration. The field of tissue engineering has largely defined this evolutionary leap. One active area of investigation is the development of tissue engineering strategies for adipose tissue. Bioengineers, life scientists, and reconstructive surgeons are synergistically coupling expertise in areas such as cell culture technology, tissue transfer, cell differentiation, angiogenesis, computer modeling, and polymer chemistry to regenerate adipose tissue de novo for breast replacement and soft-tissue augmentation following tumor resection. This work presents the current state of the art in adipose tissue engineering, as well the clinically translatable strategies currently under development. *Semin. Surg. Oncol. 19:302–311, 2000.*

KEY WORDS: **adipose tissue; biomedical engineering; breast implantation; capillaries; mastectomy; reconstructive surgical procedures**

INTRODUCTION

A mastectomy results in the loss of the breast mound that is largely composed of adipose tissue. Ideally, a surgeon prefers to refill the breast envelope with a patient's own adipose tissue. The contemporary standard of care for breast reconstruction includes utilizing implants or tissue transfer, both of which possess limitations [1]. Resection of tumors in the head and neck, and upper and lower extremities often results in contour defects due to loss of soft tissue, which is largely composed of subcutaneous adipose tissue. Again, a surgeon prefers to use a patient's own adipose tissue to sculpt contour deformities. As illustrated in Table I, there are numerous reconstructive, cosmetic, and correctional indications for the development of a clinically translatable strategy with which to restore a volume of adipose tissue.

Tissue engineering, coupled with knowledge gleaned from obesity and diabetes research as well as the amassed clinical experience with fat grafting, possesses the potential to provide surgeons with a source of patient-specific adipose tissue of a predefined volume. This work explores the current state of the art in adipose tissue engineering. The three fundamental components of a tissue construct—cells, scaffold, and microenvironment—are discussed first. Readers desiring a comprehensive perspective of tissue engineering should refer to the texts by Patrick et al. [2] and Lanza et al. [3]. Finally, strategies proposed for adipose tissue engineering germane to oncologic reconstructive surgery are presented.

CELLS

Fat Cells

Adipose tissue is ubiquitous in the human body; it is the largest tissue in the body, and is uniquely expendable in that most patients possess excess that can be harvested without creating contour deformities. However, autologous fat transplantation yields poor results, with 40–60% reduction in graft volume [1,4]. The reduction in adipose volume is postulated to be related to insufficient re-

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TABLE I. Indications for a Tissue Engineered Adipose Strategy

Category	Application	Specifics	Incidence or number of procedures/year
Reconstructive	Oncologic resection	Mastectomies	69,683 Breast reconstructions (3.1%)[a] [39]
		Parotidectomies	
	Complex trauma	Soft tissue deficits	
	Congenital abnormalities	Hemifacial microsomia	1 in 4,000 to 1 in 5,000 [40]
		Poland's syndrome	1 in 20,000 to 1 in 32,000 [41]
		Romberg's syndrome	
Cosmetic	Augmentation	Breast	132,378 Breast augmentations (augmentation mammoplasty) (6.0%)[a] [39]
			31,525 Breast lifts (mastoplexy) (1.4%)[a] [39]
		Cheek, chin, jaw	2,864 Cheek implants (malar augmentation) (0.1%)[a]
			4,795 Chin augmentations (mentoplasty) (0.2%)[a] [39]
		Lips	
		Buttocks	
	Rejuvenation	Wrinkles	1,246 Buttock lifts (0.1%)[a] [39]
			1,463 Fibril injections (0.1%)[a] [39]
	Nonspecific revision/resculpting	Various locations	45,851 Collagen injections (2.1%)[a] [39]
			25,437 Fat injections (1.1%)[a] [39]
Correctional	Implant removal	Breast	43,681 Removals (2.0%)[a]
	Bulking agent	Stress urinary incontinence	1,500,000
		Vocal cord insufficiency	
	Orthotic-related	Atrophied "cushion" in ball/heel of aged foot	
	Augmentation	Soft tissue deficits	

[a]Data represent 1998 statistics and (%) denotes the percentage of total plastic surgery procedures represented by the data.

vascularization. Some success has been achieved in transplanting small volumes of fat where diffusion can support cell survival [5,6]. The small volumes, however, are not clinically relevant for oncologic defects. The advent of liposuction led investigators to attempt the use of single-cell suspensions of mature adipocytes for soft-tissue augmentation. However, since they possess a cytoplasm composed of 80–90% lipid, aspirated adipocytes are easily traumatized by the mechanical forces of liposuction, resulting in ~90% damaged cells. The remaining 10% tend to form cysts or localized necrosis postinjection. Moreover, the terminal phenotype of mature adipocytes precludes taking advantage of ex vivo cell culture technology.

Recent progress has been made using preadipocytes—precursor cells that differentiate into mature adipocytes. Preadipocytes are fibroblast-like cells that uptake lipid during differentiation (Fig. 1). They grow easily with standard cell culture technologies and can be expanded ex vivo, and the molecular biology involved in preadipocyte differentiation has largely been elucidated through active research in the obesity and diabetes regimes [7–9]. However, much of the application-based biology of preadipocytes remains unknown (e.g., cell adhesion, cell motility, and response to various microenvironments). Human, rat, and swine preadipocytes have been routinely cultured [10–15]. Preadipocytes are typically isolated from enzyme-digested adipose tissue or liposuction aspirates. One can envision obtaining preadipocytes during a preoperative visit, for example, using current outpatient liposuction techniques. Alternatively, adipocyte stem cells may potentially allow one to develop cultures of preadipocytes. Researchers are predominantly focusing on using subcutaneous preadipocytes for tissue engineering strategies. It is known that fat depots at different anatomical locations behave differently [16–19]. Hence it remains to be seen if subcutaneous preadipocytes can adequately replace mammary adipose tissue. They should, however, suffice for soft-tissue augmentation strategies. The effects of patient age and menopausal status on preadipocyte biology within tissue-engineered constructs remain elusive. Moreover, many oncologic patients experience pre- or postoperative radiotherapy and/or chemotherapy. The effect of adjuvant therapies on adipose tissue engineering is presently unknown.

Vascular Cells

Any potential clinically translatable tissue engineering modality must consider the microvasculature. Clinical experience with tissue transfer alone proves this point. Adipose tissue is unique in that it possesses the capacity to continue to grow, and its vascular network grows in tandem (i.e., de novo angiogenesis) [20]. Adipose tissue is highly vascular. In fact, it is reported that each adipocyte is attached to at least one capillary. The capillary density of adipose is approximately one-third that of muscle. However, from a metabolic standpoint, normalizing for active protoplasm (since an adipocyte is largely lipid within its cytoplasm), the capillary bed of adipose is far richer (by ~2–3×) than that of muscle. Hence, it is paramount that adipose tissue

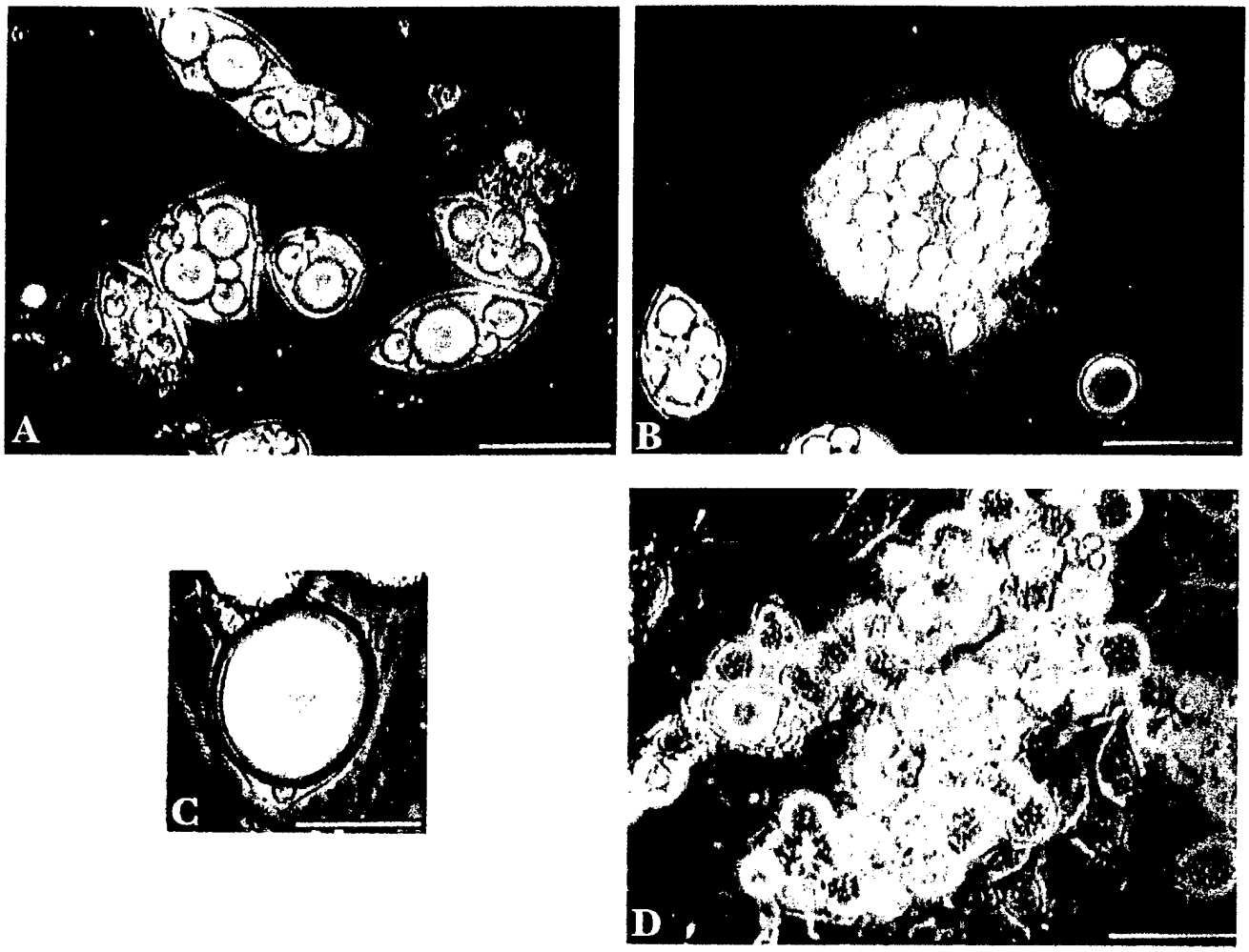

Fig. 1. Growth and differentiation of rat preadipocytes. Images **A** and **B** are phase contrast images of preadipocytes 7 days after initiation of differentiation. Note the accumulation and coalescence of lipid pools. **C:** Image of unilocular lipid pool within a single preadipocyte. **D:** Lobules of adipose tissue formed in vitro. Bars denote 50 μm.

constructs be well vascularized. In addition, adipose tissue is known to enhance angiogenesis through the secretion of growth factors and extracellular matrices (ECMs) [21–23].

Of the three biological mechanisms available to vascularize a tissue equivalent, only two are available to adults: revascularization and inosculation. Revascularization denotes the growth of capillaries from a host site into a tissue equivalent. Except for relatively thin constructs that can survive by diffusion, the slow kinetics (on the order of weeks) of this process abrogates its use for large constructs. It is hypothesized that strategies for soft-tissue augmentation will rely on revascularization since relatively thin layers of adipose will be required. In addition, wrapping a construct with highly vascular tissue may enhance revascularization. It has been proposed to use the highly vascular and adipocyte rich omentum to encase constructs [24,25]. Inosculation is the process of two capillaries or capillary networks fusing together. The kinetics of inosculation occur on the order of hours and are the predominant factor in reconstructive surgeons' ability to transfer tissue from a donor site to a recipient site. The use of inosculation in a tissue engineering strategy requires either the seeding of microvascular endothelial cells into a tissue equivalent followed by in situ development of a capillary network, or the ex vivo development and implantation of a capillary network. Strategies for breast reconstruction will no doubt require capillary networks as the large tissue mass required precludes relying solely on revascularization. Both modalities are actively being investigated but are hindered by the lack of understanding of the biological mechanisms that control inosculation, capillary formation, and cell culture technology of microvascular endothelial cells.

SCAFFOLDS

Numerous natural, synthetic, and hybrid materials have been utilized to act as adipose surrogates (Table II). These

TABLE II. Materials Used in Place of Adipose Tissue in Reconstructive Surgery

Material	Product (vendor)		Primary component(s)
ECM/Tissue Matrix	AlloDerm®	(LifeCell Corp)	Decellularized human dermal tissue
	Autologen®	(Collagenesis Inc)	Autologous human dermal collagen
	Cymetra™	(LifeCell Corp)	Micronized AlloDerm®
	Dermalogen™	(Collagenesis Inc)	Allogenous human dermal tissue matrix
	Fibrel®	(Mentor Corp)	Fibrin gel
	Hylaform®	(Biomatrix Corp)	Hyaluronic acid gel
	Restylane™	(Q-Med)	Viscoelastic hylan gel
	Tisseel®	(Baxter)	Human fibrin
	Zyderm® I	(Collagen Aesthetics)	Bovine dermal collagen (35 mg/mL)
	Zyderm® II	(Collagen Aesthetics)	Bovine dermal collagen (65 mg/mL)
	Zyplast®	(Collagen Aesthetics)	Zyderm® II with glutaraldehyde
Mineral/vegetable/oils			
Paraffin			
Polymers	Artecoll®	(Fofil Medical Int)	Polymethylmethacrylate microspheres
	Bioplastique®	(Bioplasty Inc)	Cross-inked polydimethylsiloxane
	Gortex®	(W.L. Gore & Associates)	Expanded polytetrafluoroethylene (ePTFE)
	Marlex®	(Davol Inc)	Porous/mesh-form polyethylene
	Softform™	(Collagen Aesthetics)	Expanded polytetrafluoroethylene (ePTFE)
Silicone	Various formulas	(Dow Corning)	

LifeCell Corporation, Branchburg, NJ; Collagenesis, Inc., Beverly. MA; Mentor Corp., Santa Barbara, CA; Biomatrix Corp., Ridgefield, NJ: Q-Med, Uppsala, Sweden; Collagen Aesthetics, Palo Alto, CA; Rofil Medical Int., Laguna Beach, CA; Bioplasty, Inc., Maastricht, The Netherlands; W.L. Gore & Associates, Flagstaff, AZ; Davol, Inc., Cranston, RI; Dow Corning. Midland, MI.

have predominantly been used to replace adipose volume and not function; that is, surgeons have largely focused on filling a defect site. Adipose tissue engineering possesses the potential to restore both volume and function. This depends, however, on preadipocytes adhering to appropriate support structures, or scaffolds. A support structure is required for anchorage-dependent cells to migrate and proliferate and to give a tissue equivalent the boundary conditions for final overall tissue shape. Scaffolds may either be implanted or injected.

Implantable materials utilized for adipose tissue engineering have predominantly been porous biodegradable polymer foams [1,4,26]. For instance, PLGA scaffolds preseeded with preadipocytes have demonstrated adipose tissue formation [26]. Polymer foams, however, will probably not be the optimum choice for breast scaffolds as they are too rigid for the breast envelope and would be uncomfortable for the patient. Nonbiodegradable scaffolds have also been investigated. Kral and Crandall [27] recently demonstrated the attachment and proliferation of preadipocytes on fluorotex monofilament-expanded polytetrafluoroethylene scaffolds coated with various ECMs.

Injectable materials, such as hydrogels, inherently possess optimum mechanical properties for use in the breast envelope, and for injection into soft-tissue defects. Both alginate and hyaluronic acid gels have been investigated [4,28,29]. In addition, preadipocytes proliferate and differentiate within fibrin gels (Fig. 2).

The optimum scaffold for breast tissue engineering remains elusive. Derivatizing polymers with adhesion molecules can potentially optimize scaffolds. However, this strategy is complicated by the fact that the constitution and distribution of the ECMs varies during adipocyte differentiation [30]. We have shown that preadipocytes readily adhere to laminin and fibronectin (Fig. 3). Hence, it would

Fig. 2. Rat preadipocytes seeded into and differentiating within a 2-mm-thick fibrin gel.

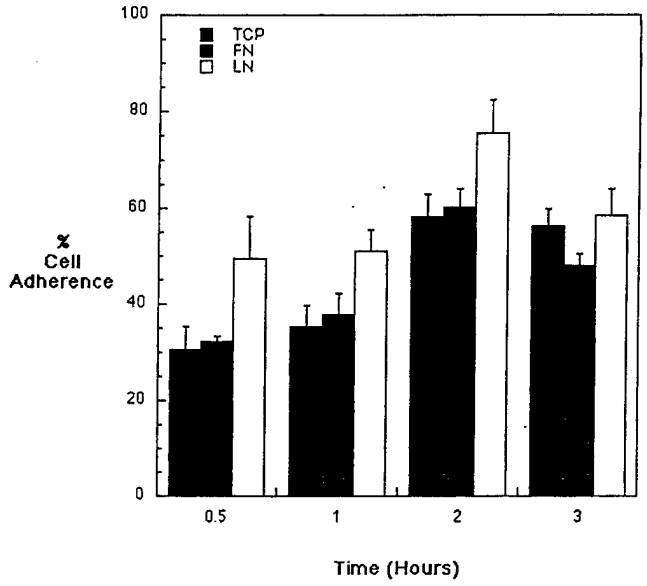

Fig. 3. Kinetics of preadipocyte adherence to tissue culture plastic (TCP), laminin (LN, 9–11 μg/cm^2), and fibronectin (FN, 5–7 μg/cm^2). Data denote mean ± SD.

seem conceivable to derivatize scaffolds with YGSIR and RGD, the cell-binding sequences of laminin and fibronectin, respectively. In fact, Mooney and colleagues [28] have derivatized alginate with RGD and observed increased adipogenesis when injected in a rat model. Although short-term studies have demonstrated adipose formation within biodegradable polymers [26], it remains to be determined whether the formed adipose tissue resorbs over the long term. We are currently involved in a year-long study to determine the sustainability of tissue-engineered adipose tissue (unpublished data).

TABLE III. Factors Positively (+) or Negatively (–) Affecting Adipose Differentiation

Factor	Effect
aFGF	+/–
bFGF	+/–
EGF	–
Glucocorticoid	+
Growth hormone	+
IGF-1	+
IL-11	–
Insulin	+
Interferon-γ	–
PDGF	+/–
Prostaglandins	+
TGF-α	–
TGF-β	–
Thyroid hormone	+
TNF-α	–

MICROENVIRONMENT

The microenvironment surrounding a tissue construct affects its differentiation and rate of tissue formation. Adipogenesis can be affected, in part, by growth factors (endo- and exogenous), pO_2 (normoxia vs. hypoxia), pH, adhesion molecules on ECM and support cells, and micromotion. Table III illustrates chemical factors that are reported to affect preadipocyte differentiation into adipocytes. Some factors (e.g., aFGF, bFGF, and PDGF) elicit conflicting results due to the fact it has not been elucidated whether the factors affect preadipocyte differentiation directly or indirectly through angiogenesis. Kawaguchi et al. [31] demonstrated de novo adipogenesis following injection of Matrigel and bFGF in mice. Yuksel and colleagues [32,33] have used biodegradable microspheres loaded with insulin, bFGF, and IGF-1 to

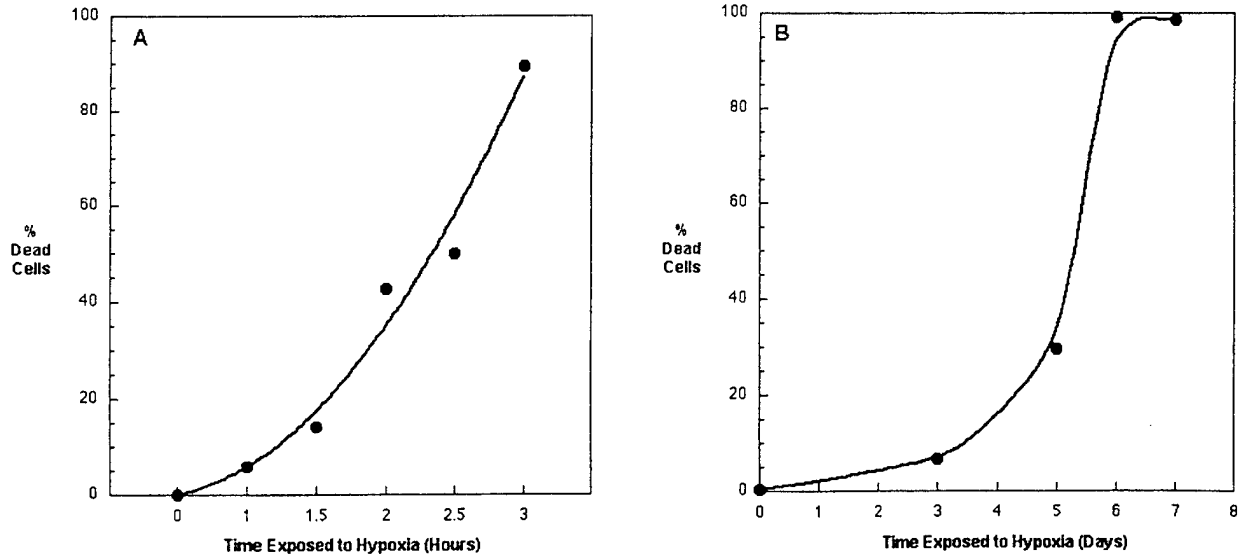

Fig. 4. Effect of hypoxia on the in vitro viability of (A) preadipocytes and (B) endothelial cells.

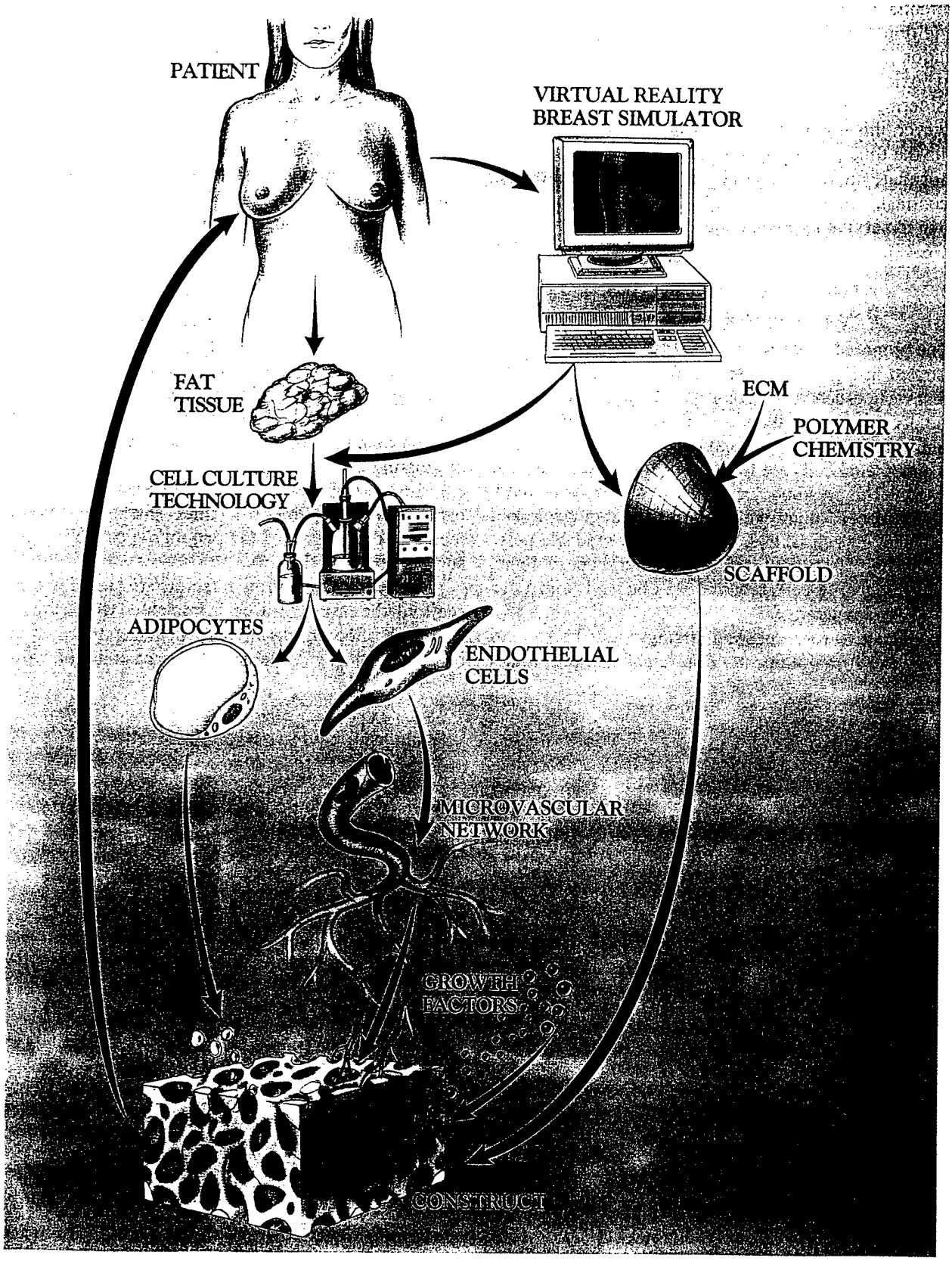

Fig. 5. Overview of proposed adipose tissue engineering strategy for developing a de novo breast mound.

differentiate preadipocytes to mature adipocytes in vivo. In addition, both EGF and TNF-α inhibit adipose differentiation [34,35]. Preadipocytes are extremely sensitive to hypoxic environments (Fig. 4). This is not surprising considering the historical results of free-fat grafting. However, this highlights a major design constraint as it limits the time preadipocytes can be implanted without an adequate microvascular network to supply nutrients. In contrast, microvascular endothelial cells have been shown to survive hypoxic conditions for 5–7 days (Fig. 4) [36,37].

PROPOSED STRATEGIES

Breast Reconstruction Strategies

One proposed strategy for breast tissue engineering is illustrated in Fig. 5. Adipose tissue is obtained from the patient via liposuction or fat biopsy. From the tissue sample, preadipocytes and capillary endothelial cells are isolated via enzymatic digestion and expanded ex vivo. The capillary endothelial cells are finessed to form microvascular networks. The preadipocytes and microvascular network are later placed, along with appropriate angiogenic and adipogenic growth factors, within a biodegradable polymer scaffold. The patient-specific scaffold shape, volume, and the number of cells required are obtained from a virtual breast simulator.

A breast tissue engineering strategy must be patient-specific to be truly clinically translatable. Unlike strategies for organs that can, for the most part, be grown as "one size fits all," breast shape and volume vary widely among the patient population. Breast implants, for instance, range from 100 mL to 2 L. Hence, methods must exist to predetermine design parameters preoperatively such that the final outcome is known a priori. To accomplish this goal, bioengineers, physicians, and computer scientists have combined skill sets to develop a first-generation virtual reality breast simulator (Fig. 6). The current system uses a global parametric deformable model of an ideal breast, and allows the surgeon to manipulate the shape of the breast by varying five key shape variables, analogous to the aesthetic and structural elements surgeons inherently vary during breast reconstruction. The variables include ptosis (sagging of the breast), top-shape (the breast's upper concavity/convexity), turn-top (orientation of the top half of the breast with respect to the shoulders), flatten-side (the side's concavity/convexity), and turn (deflection of nipple orientation from a perpendicular axis originating at the chest wall). The second generation of the virtual reality model is being developed to be truly patient-specific by importing three-dimensional measurements of the surface of the patient's breast obtained via surface scanning (Fig. 6).

Once the cells and growth factors are placed within the scaffold, the entire construct is implanted within the breast envelope following mastectomy. Using microsurgery techniques, the host vascular system is anastomosed with the

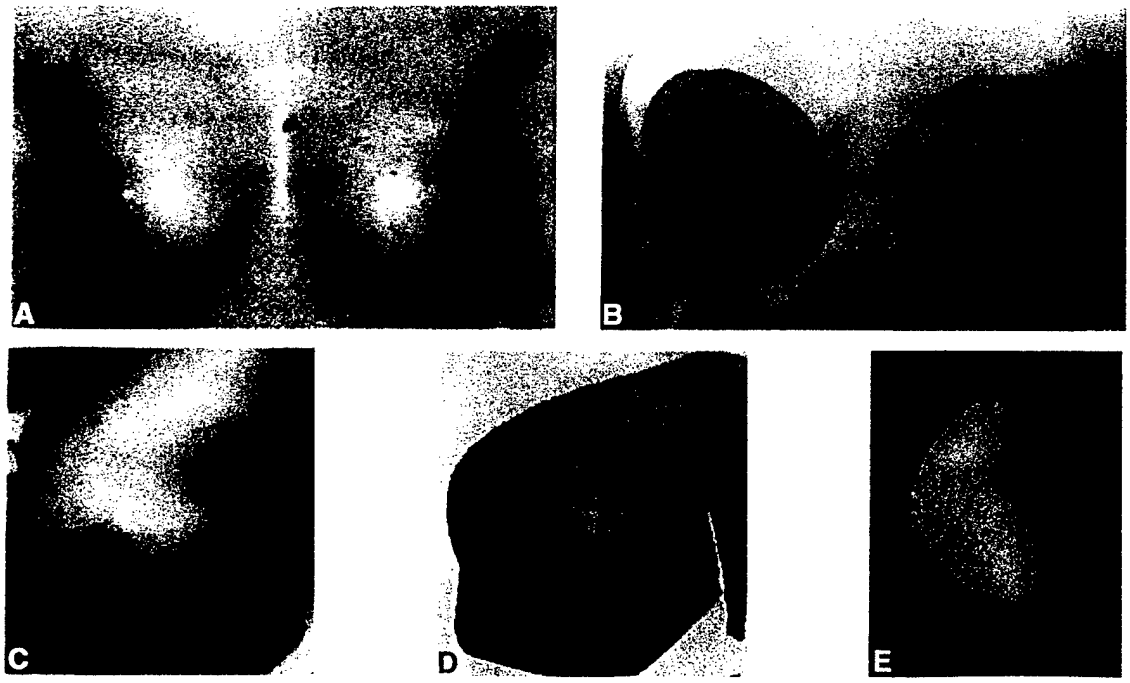

Fig. 6. Development of a virtual reality breast simulator. **A:** Image of patient. **B:** Virtual breast designed by the physician based on image A, using the first-generation simulator. **C:** Image of patient breast. **D:** 3D surface scan of breast in image C. **E:** Fitted virtual breast model of breast in C, resulting in a required volume of 987 mL and surface area of 453 cm^2. Images acquired in collaboration with Dr.M.J. Miller (M.D. Anderson Cancer Center) and Dr. I. Kakadiaris (University of Houston).

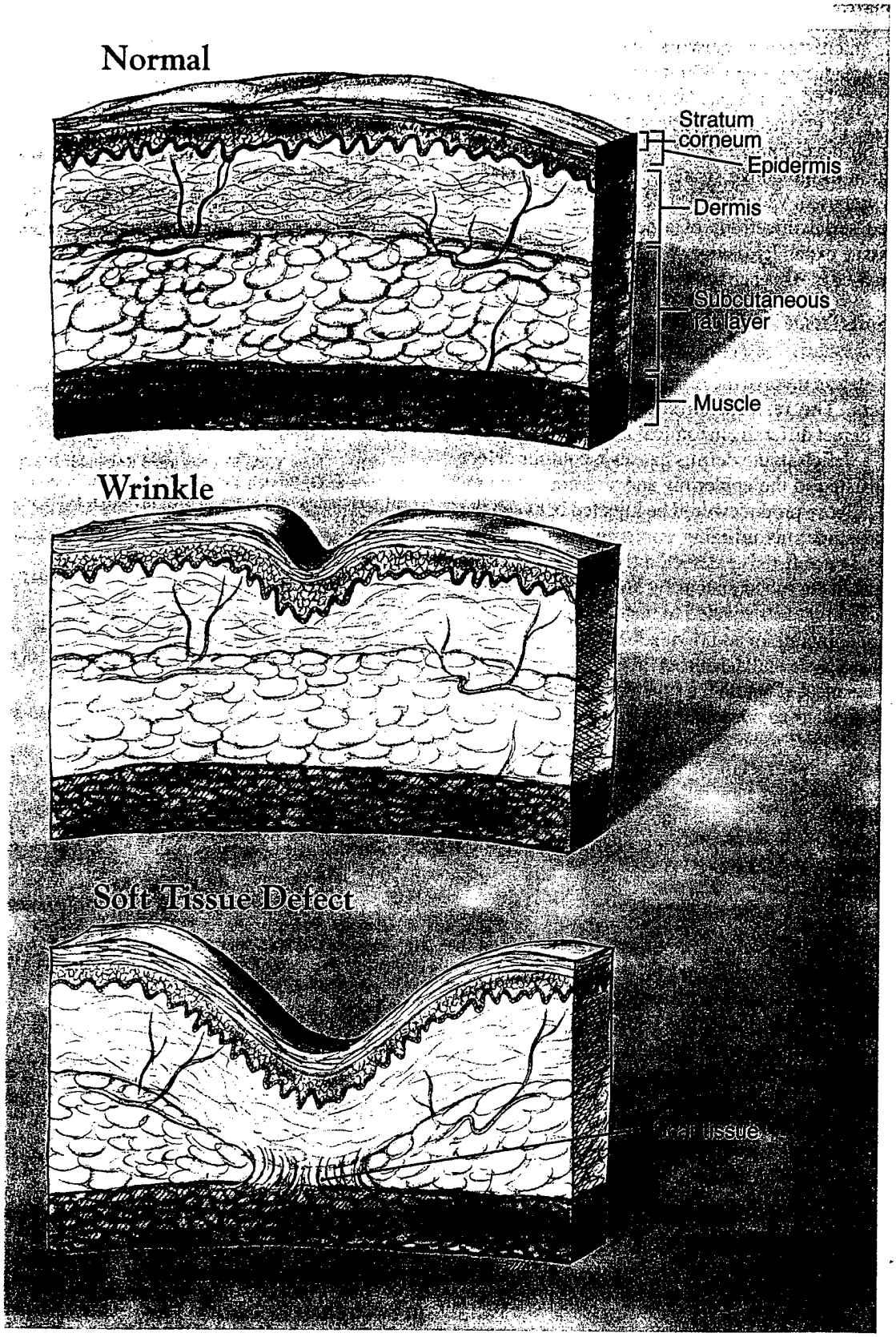

Fig. 7. Organizational differences between a normal cross-section of skin and that of a wrinkle and soft-tissue defect. In a wrinkle, a deficit in the dermis causes a contour defect that is translated to the epidermis and stratum corneum. In a soft-tissue defect, a tissue deficit occurs much deeper in the subcutaneous fat layer, causing a large contour defect that is translated to the dermis, epidermis, and stratum corneum. In addition, adhesion plaques form between the muscle and dermis.

construct's microvascular network. As the scaffold degrades, the preadipocytes proliferate and differentiate into adipose tissue. The microvascular system will reorganize accordingly. In essence, the patient becomes her own bioreactor for developing a new breast.

In addition to the above strategy, adjustable implants have been proposed. Vacanti and colleagues [38] have conceptualized serial injections of a cell-seeded hydrogel within a tissue expander device, with the tissue expander being decreased in size each time an injection is conducted.

Soft-Tissue Augmentation Strategies

Tissue engineering strategies for wrinkle and soft-tissue augmentation involve the restoration of localized contour defects. The restoration site for a wrinkle and soft-tissue defect differ in anatomical location (Fig. 7). In a wrinkle, a deficit in the dermis causes a contour defect that is translated to the epidermis and stratum corneum. Hence, an adipose product would be injected between the dermis and epidermis utilizing contemporary injection techniques. In a soft-tissue defect, a tissue deficit occurs much deeper in the subcutaneous fat layer, causing a large contour defect that is translated to the dermis, epidermis, and stratum corneum. In addition, adhesion plaques form between the muscle and dermis. Hence, an adipose product would be injected within the subcutaneous fat between the muscle and dermis. During the injection, the adhesion plaques would have to be cleaved. Obvious strategies include injecting hydrogels containing preadipocytes and adipogenic/angiogenic factors. Other strategies may involve developing a thin, flexible fabric composed of a biodegradable polymer or polymer blend that can be preseeded with preadipocytes.

CONCLUSIONS

The field of tissue engineering offers great potential in abrogating the current limitations of breast reconstruction and soft tissue augmentation following tumor resection. To be sure, great strides have already been achieved. However, the continued progress, and ultimately the clinical translation, of adipose tissue engineering applied to oncologic reconstructive surgery will require the active, synergistic collaboration of bioengineers, life scientists, and oncologic reconstructive surgeons. Only then will we truly be able to take advantage of technology and scientific knowledge to increase patient quality of life.

REFERENCES

1. Patrick Jr CW, Chauvin PB, Robb GL: Tissue engineered adipose. In Patrick Jr. CW, Mikos AG, McIntire LV (eds): "Frontiers in tissue engineering." Oxford: Elsevier Science; 1998; p. 369–382.
2. Patrick Jr CW, Mikos AG, McIntire LV: "Frontiers in tissue engineering," Oxford: Elsevier Science; 1998.
3. Lanza RP, Langer R, Vacanti J: "Principles of tissue engineering." San Diego: Academic Press: 2000.
4. Lee KY, Halberstadt CR, Holder WD, Mooney DJ: Breast reconstruction. In Lanza RP, Langer R, Vacanti J (eds): "Principles of tissue engineering." San Diego: Academic Press; 2000; p. 409–423.
5. Coleman SR: Long-term survival of fat transplants: controlled demonstrations. Aesth Plast Surg 1995;19:421–425.
6. Coleman SR: Facial recontouring with lipostructure. Clin Plast Surg 1997;24:347–367.
7. Mandrup S, Lane MD: Regulating adipogenesis. J Biol Chem 1997;272:5367–5370.
8. Loftus TM, Lane MD: Modulating the transcriptional control of adipogenesis. Curr Opin Genet Devel 1997;7:603–608.
9. MacDougald OA, Lane MD: Transcriptional regulation of gene expression during adipocyte differentiation. Ann Rev Biochem 1995;64:345–73.
10. Entenmann G, Hauner H: Relationship between replication and differentiation in cultured human adipocyte precursor cells. Am J Physiol 1996;270:C1011–C1016.
11. Hausman GJ, Richardson RL: Newly recruited and pre-existing preadipocytes in cultures of porcine stromal-vascular cells: morphology, expression of extracellular matrix components, and lipid accretion. Anim Sci 1998 1998;76:48–60.
12. Novakofski JE: Primary cell culture of adipose tissue. In Hausman GJ, Martin RJ (eds): "Biology of the adipocyte: research approaches." New York: Van Nostrand Reinhold; 1987; p. 160–197.
13. Shillabeer G, Forden JM, Lau DCW: Induction of preadipocyte differentiation by mature fat cells in the rat. J Clin Invest 1989;84: 381–387.
14. Strutt B, Khalil W, Killinger D: Growth and differentiation of human adipose stromal cells in culture. In Jones GE (ed): "Methods in molecular medicine: human cell culture protocols." Totowa, NJ: Human Press Inc.; 1996; p. 41–51.
15. Van RLR, Roncari DAK: Isolation of fat cell precursors from adult rat adipose tissue. Cell Tiss Res 1977;181:197–203.
16. Djian P, Roncari DAK, Hollenberg CH: Influence of anatomic site and age on the replication and differentiation of rat adipocyte precursors in culture. J Clin Invest 1983;72:1200–1208.
17. Hauner H, Entenmann G: Regional variation of adipose differentiation in cultured stromal-vascular cells from the abdominal and femoral adipose tissue of obese women. Int J Obesity 1991;15:121–126.
18. Kirkland JL, Hollenberg CH, Kindler S, Gillon WS: Effects of age and anatomic site on preadipocyte number in rat fat depots. J Gerontol 1994;49:B31–B35.
19. Kirkland JL, Hollenberg CH, Gillon WS: Effects of fat depot site on differentiation-dependent gene expression in rat preadipocytes. Int J Obes Rel Metabol Disord 1996;20 Suppl 3:S102–S107.
20. Crandall DL, Hausman GJ, Kral JG: A review of the microcirculation of adipose tissue: Anatomic, metabolic, and angiogenic perspectives. Microcirculation 1997;4:211–232.
21. Varzaneh FE, Shillabeer G, Wong KL, Lau DCW: Extracellular matrix components secreted by microvascular endothelia cells stimulate preadipocyte differentiation in vitro. Metabolism 1994;43(7): 906–912.
22. Silverman KJ, Lund DP, Zetter BR, et al: Angiogenic activity of adipose tissue. Biochem Biophys Res Commun 1988;153:347–352.
23. Zhang QX, Magovern CJ, Mack CA, et al: Vascular endothelial growth factor is the major angiogenic factor in omentum: mechanism of the omentum-mediated angiogenesis. J Surg Res 1997; 67:147–154.
24. Erol OO, Spira M: Reconstructing the breast mound employing a secondary island omental skin flap. Plast Reconstr Surg 1990;86: 510–518.
25. Sugawara Y, Harii K, Yamada A, et al: Reconstruction of skull defects with vascularized omentum transfer and split calvarial bone graft: two case reports. J Reconstr Microsurg 1998;14:101–108.
26. Patrick Jr CW, Chauvin PB, Reece GP: Preadipocyte seeded PLGA scaffolds for adipose tissue engineering. Tissue Eng 1999;5:139–151.
27. Kral JG, Crandall DL: Development of a human adipocyte synthetic polymer scaffold. Plast Reconstr Surg 1999;104:1732–1738.
28. Marler JJ, Guha A, Rowley J, et al: Soft-tissue augmentation with injectable alginate and syngeneic fibroblasts. Plast Reconstr Surg 2000;105:2049–2058.
29. Duranti F, Salti G, Bovani B, et al: Injectable hyaluronic acid gel for soft tissue augmentation. Dermatol Surg 1998;24:1317–1325.

30. Kubo Y, Kaidzu S, Nakajima I, et al: Organization of extracellular matrix components during differentiation of adipocytes in long-term culture. In Vitro Cell Dev Biol 2000;36:38–44.
31. Kawaguchi N, Toriyama K, Nicodemou-Lena E, et al: De novo adipogenesis in mice at the site of injection of basement membrane and basic fibroblast growth factor. Cell Biology 1998;95:1062–1066.
32. Yuksel E, Weinfeld AB, Cleek R, et al: Increased free fat-graft survival with the long-term, local delivery of insulin, insulin-like growth factor-I, and basic fibroblast growth factor by PLGA/PEG microspheres. Plast Reconstr Surg 2000;105:1712–1720.
33. Yuksel E, Weinfeld AB, Cleek R, et al: De novo adipose tissue generation through long-term, local delivery of insulin and insulin-like growth factor-1 by PLGA/PEG microspheres in an *in vivo* rat model: a novel concept and capability. Plast Reconstr Surg 2000; 105:1721–1729.
34. Serrero G: EGF inhibits the differentiation of adipocyte precursors in primary cultures. Biochem Biophys Res Commm 1987;146: 194–202.
35. Kras KM, Hausman DB, Martin RJ: Tumor necrosis factor-α stimulates cell proliferation in adipose tissue-derived stromal-vascular cell culture: promotion of adipose tissue expansion by paracrine growth factors. Obesity Res 2000;8:186–193.
36. Dore-Duffy P, Balabanov R, Beaumont T, et al: Endothelial activation following prolonged hypobaric hypoxia. Microvasc Res 1999;57:75–85.
37. Shweiki D, Itin A, Soffer D, Keshet E: Vascular endothelial cell growth factor induced by hypoxia may mediate hypoxia-initiated angiogenesis. Nature 1992;359:843–845.
38. Vacanti JP, Atala A, Mooney DJ, Langer RS: Breast tissue engineering. US Patent 5,716,404. USA, 1998.
39. American Society of Plastic Surgeons and Plastic Surgery Education Foundation; [1998; cited 00 Oct 17]. Available from: http://www.plasticsurgery.org/.
40. Kearns GJ, Padwa BL, Mulliken JB, Kaban LB: Progression of facial asymmetry in hemifacial microsomia. Plast Reconstr Surg 2000;105:492–498.
41. Havlik RJ, Sian KU, Wagner JD, et al: Breast cancer in Poland syndrome. Plast Reconstr Surg 1999;104:180–182.

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Preadipocyte Seeded PLGA Scaffolds for Adipose Tissue Engineering

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ABSTRACT

Adipose tissue equivalents have not been addressed as yet despite the clinical need in congenital deformities, posttraumatic repair, cancer rehabilitation, and other soft tissue defects. Preadipocytes were successfully harvested from rat epididymal fat pads of Sprague-Dawley and Lewis rats and expanded *ex vivo*. *In vitro* cultures demonstrated full differentiation of preadipocytes into mature adipocytes with normal lipogenic activity. The onset of differentiation was well-controlled by regulating preadipocyte confluency. Poly(lactic-co-glycolic) acid (PLGA) polymer disks with 90% porosity, 2.5 mm thick, 12 mm diameter, pore size range of 135–633 μm were fabricated and seeded with preadipocytes at 10^5 cells/mL. Disks *in vitro* demonstrated fully differentiated mature adipocytes within the pores of the disks. Short-term *in vivo* experiments were conducted by implanting preseeded disks subcutaneously on the flanks of rats for 2 and 5 weeks. Histologic staining of harvested disks with osmium tetroxide (OsO_4) revealed the formation of adipose tissue throughout the disks. Fluorescence labeling of preadipocytes confirmed that formed adipose tissue originated from seeded preadipocytes rather than from possible infiltrating perivascular tissue. This study demonstrates the potential of using primary preadipocytes as a cell source in cell-seeded polymer scaffolds for tissue engineering applications.

INTRODUCTION

TISSUE ENGINEERING is a broad multidisciplinary field dedicated to the advancement of the human biological environment in healing, correction of deformity, as well as overall tissue function. A natural next step for soft tissue structuring efforts lies in the area of adipose tissue. As early as 1893,[1] surgeons have attempted to transplant autologous adipose tissue with minimal success.[2–7] Conventional procedures for reconstructing breast or other soft tissue defects composed of adipose tissue involve "robbing Peter to pay Paul." That is, a section of living tissue that carries its own blood supply (donor site) is moved from one area of the body to another to repair a deficit of skin, fat, muscle, and to restore movement, or skeletal support. Often, muscle must be used for the most extensive soft tissue reconstruction requirements.[10] Composite flaps comprised of muscle, fat, and skin restore similar consistency and permit the transposition

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of relatively large volumes of tissue for reconstruction. However, there are several problems associated with use of muscle for soft tissue reconstruction, such as loss of some function at the donor site, aberrant degree of "softness" at the recipient site, and decrease in soft tissue volume with time because of muscle atrophy (25–50%) due to noninnervation.

Adipose tissue *in vivo* is composed of mature adipocytes that are extremely fragile and do not proliferate.[8,9] Moreover, unless a blood supply is immediately incorporated with the transplanted mature adipose tissue (or fat) the tissue begins to undergo necrosis. These limitations provide challenges for developing viable fat tissue solutions for current reconstructive applications in soft tissue augmentation and, ultimately, for incorporation into composite flap tissue for clinical use to increase soft tissue bulk and help create or repair appropriate superficial body contour and shape where well-vascularized soft tissue is needed.

There are extensive indications for the use of adipose tissue equivalents, primarily for reconstructive purposes in congenital deformities (e.g., Poland's syndrome, Romberg's syndrome, and hemifacial microsomia), posttraumatic repair, and cancer rehabilitation. As a first step toward developing adipose tissue equivalents, poly(lactic-co-glycolic) acid (PLGA) polymer disks have been fabricated and seeded with preadipocytes. Preadipocytes *in vitro* and *in vivo* differentiate into mature adipocytes and exhibit lipogenic activity. Histology of seeded PLGA disks implanted subcutaneously in rats demonstrates the formation of adipose tissue throughout the disks and fluorescence labeling confirms the source of adipose tissue to be the seeded preadipocytes. This study demonstrates the potential of using primary preadipocytes as a cell source in cell-seeded polymer scaffolds for tissue engineering applications.

MATERIALS AND METHODS

Adipose Harvest and in Vitro *Culture*

Adipocyte precursors (preadipocytes) are isolated from epididymal fat pads of male, 250 g, 70–80-day-old Sprague-Dawley or Lewis rats (Harlan, Indianapolis, IN) via enzymatic digestion. Briefly, rats are euthanized with CO_2 asphyxiation and the shaved harvest site is scrubbed with Betadyne followed by alcohol wash. Within 5 min of death, epididymal adipose tissue is aseptically harvested and placed in 4°C saline solution supplemented with 500 U/ml penicillin and 500 μg/ml streptomycin (Gibco, Gaithersburg, MD). Using a dissecting microscope, connective tissues and tissue containing blood vessels are resected from the fat. This minimizes fibroblast contamination of *ex vivo* cultures. Harvested tissue is finely minced with a scalpel and enzymatically digested in Ca^{+2}/Mg^{+2}-free saline supplemented with 2% (w/v) type I collagenase (Sigma Chemical Co., St. Louis, MO) and 5% (w/v) bovine serum albumin (BSA, Sigma) for 20 min at 37°C on a shaker; 5 ml of dissociation medium are required/4 fat pads. The digested tissue is filtered through a 250-μm mesh followed by a 90-μm nylon mesh to separate undigested debris and capillary fragments from preadipocytes. The filtered cell suspension is centrifuged and the resulting pellet of preadipocytes is then plated at 10^4 cells/cm^2 onto plastic culture flasks. Preadipocytes are cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS, Sigma), 100 U/ml penicillin, and 100 μg/ml streptomycin. During cell expansion, the preadipocytes are passed prior to confluency since contact inhibition initiates adipocyte differentiation and ceases preadipocyte proliferation. The 1° passage yields approximately 1.5×10^6 preadipocytes/fat pad.

Polymer Fabrication

Fabrication of 2.5-mm thick, 12-mm diameter, and 90% porosity polymer disks are prepared by a particulate-leaching technique (Figure 1).[12] Briefly, 5 g of solid 75:25 PLGA (Birmingham Polymers Inc., Birmingham, AL) polymer are dissolved in 80 ml of dichloromethane (Fisher Scientific, Pittsburgh, PA) to form a solution. Sieved NaCl crystals (Fisher) at a NaCl/PLGA weight fraction of 1:9 are evenly dispersed over a 150-mm Pyrex petri dish (Fisher) with a Teflon lining (Cole-Parmer Instrument Co., Vernon Hills, IL). The PLGA/dichloromethane solution is then gently poured over the NaCl crystals. Sieved NaCl crystal size distribution was measured with quantitative microscopy and found to be 135–633 μm. Dichloromethane is evaporated under vacuum, leaving a polymer/NaCl composite 2.5 mm thick. The composite is removed from the Teflon-lined petri dish, and 12-mm-diameter disks are cut using a plug cutter

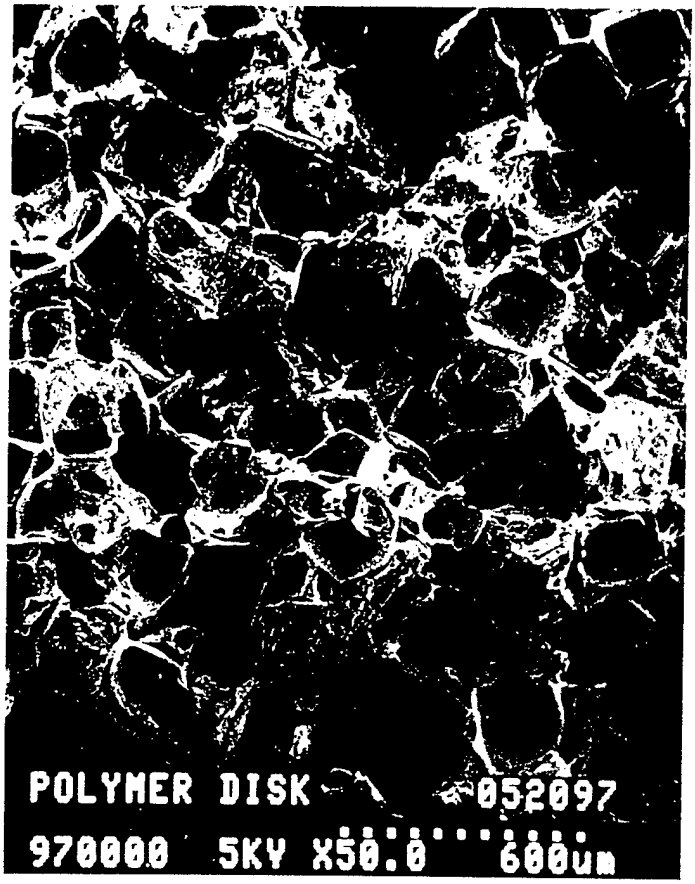

FIG. 1. SEM (×50) showing the pore structure of a PLGA disk having a pore size range of 135–633 μm, a porosity of 90%, and a 75:25 lactic-to-glycolic acid ratio. Bar = 600 μm.

and drill press. The NaCl crystals are then leached from the composite disks by immersion in 800 ml of DI water for 48 h (water changed every 8 h) to yield porous disks. Disks are lyophilized and stored in a vacuum dessicator until use. Disk diameter and thickness were chosen such that disks fit into individual wells of 24-well culture plates for *in vitro* experiments and four disks can be implanted subcutaneously in a rat for *in vivo* experiments.

Polymer Seeding

Prior to seeding, the disks are prewetted and sterilized with absolute ethanol for 30 min followed by two sterile saline washes at 20 min/wash and a DMEM wash for 20 min. A 20-μl suspension of preadipocytes (10^5 cells/ml) are injected onto each disk under sterile conditions. Prewetting permits the cell suspension to readily flow throughout the disks. Following 3 h for cell attachment, 24-well culture plates containing 1 disk/well are filled with 1.5 ml of medium/well. Medium is changed 3×/week. For *in vitro* experiments, Sprague-Dawley preadipocytes are allowed to differentiate within the disks in DMEM supplemented with 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin for periods of 14 days. For *in vivo* experiments, Lewis preadipocytes are seeded onto the disks and allowed to adhere to the polymer for a minimum of 3 h prior to implantation.

In Vivo *Implantation*

Seeded disks are implanted subcutaneously in Lewis rats under anesthesia (0.2 ml/100 gbw intramuscular injection of premixed solution composed of 64 mg/ml ketamine HCl, 3.6 mg/ml xylazine, and 0.07

mg/ml atropine sulfate). An isogenic strain is required to avoid an immune response to seeded preadipocytes. The University of Texas M.D. Anderson Cancer Center Animal Care and Use Committee has approved the implantation of adipocyte seeded disks. After shaving the back, two longitudinal incisions (~2 cm each) are made through the skin of the dorsal midline. Individual "pockets" for each disk are prepared in the subcutaneous space of both flanks by careful dissection. Disks are inserted into each pocket and sutured in place with 5-0 suture (Ethicon), as shown in Figure 2A. Two disks are placed on each side of the incision (4 disks/rat) and the incisions closed with 4-0 suture (Ethicon; Figure 2B). Animals were housed individually and fed standard rat chow. The disks are left *in vivo* 2 and 5 weeks. After the elapsed time, the rats are euthanized with CO_2 and the disks harvested. Immediately after harvest, the disks are placed in 10% neutral buffered formalin (Fisher) for future histology.

For this study, a total of 10 rats are used at 5 rats/time period. Each rat is implanted with 4 disks. Three disks are seeded with preadipocytes, and 1 disk is implanted without seeding to serve as an acellular control. A total of 30 seeded and 10 acellular disks were used in this study. Preadipocytes seeded in two rats

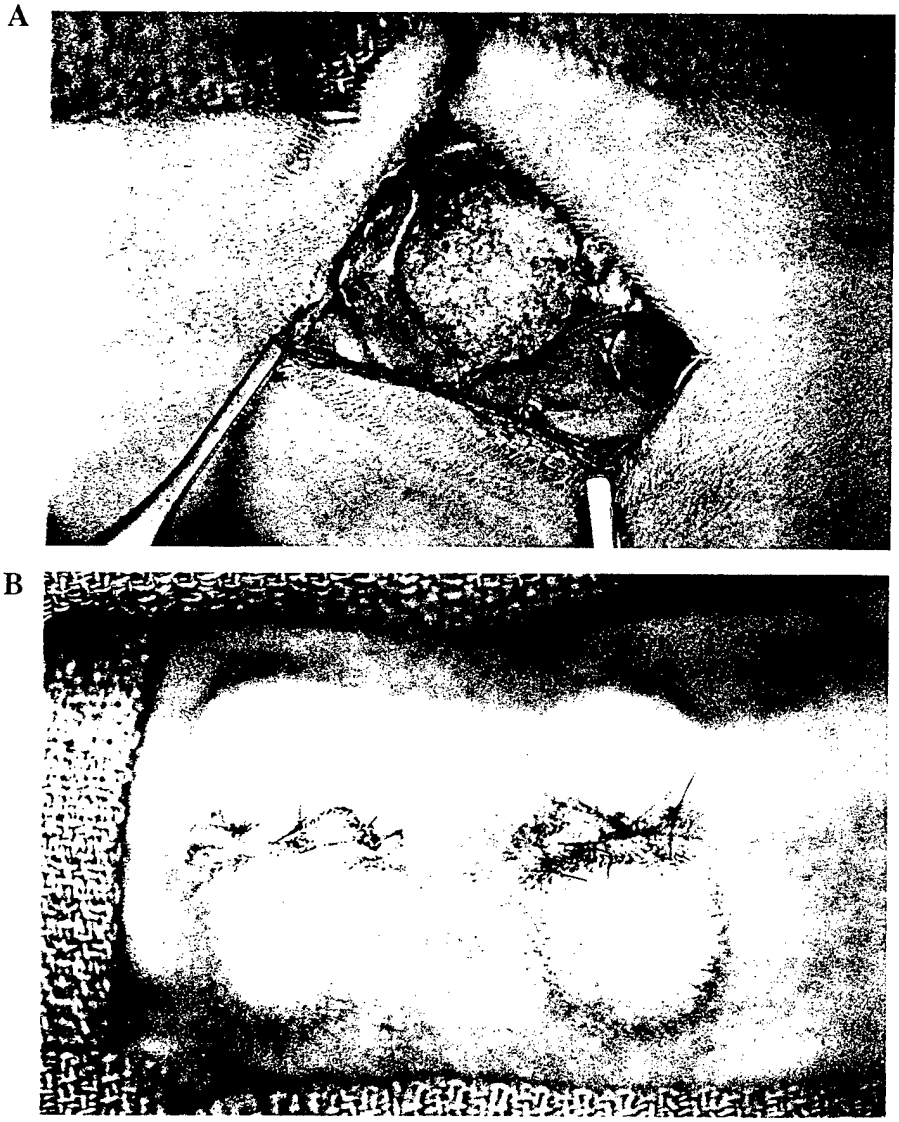

FIG. 2. Implantation of preadipocyte-seeded PLGA disks. **(A)** Disk sutured subcutaneously within prepared pouch. **(B)** Rat closed with all four disks implanted.

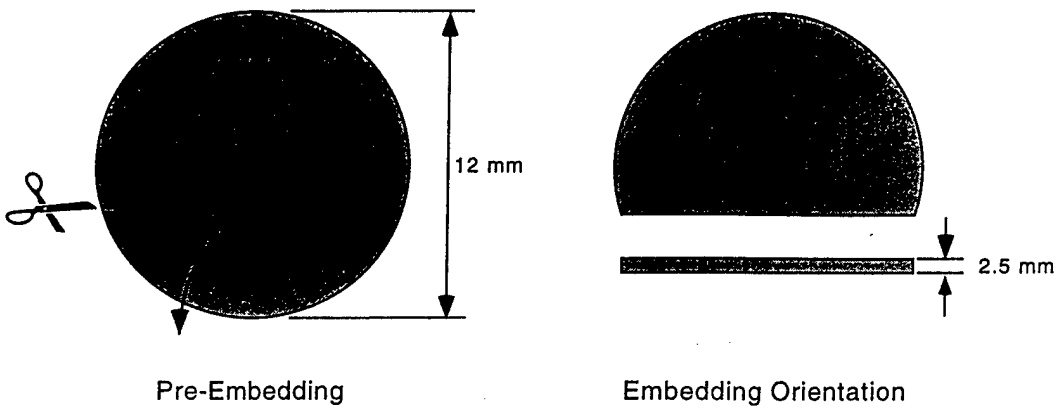

FIG. 3. Orientation of OsO_4 fixed disks for paraffin embedding. This orientation allows a face and cross-sectional cut to be made at the same time.

were prelabeled with 25 μM Hoechst 33342 (Molecular Probes, Eugene, OR) to determine if adipose formation in the disks originated from seeded cells or from surrounding perivascular tissue.

Microscopy and Digital Imaging

Cell cultures are visualized using an inverted microscope (IX 70, Olympus, Tokyo, Japan) with phase, polarized, and brightfield optics and either a B/W charged-coupled device (CCD) camera (BP500, Panasonic, Ft. Worth, TX) for gray scale images, a color CCD camera (WV-E550, Panasonic) for histology images, or a cooled CCD camera (Princeton Instruments, Trenton, NJ) for fluorescence images. The video signal from the B/W and color cameras is fed to a 24-bit, 640 × 480 pixel frame grabber (CG-7, Scion Corp., Frederick, MD) housed in a PowerTower Pro 225 computer (Power Computing Corp., Round Rock, TX) for digitization. A high-resolution monitor (SMPTE-C, JVC, Elmwood Park, NJ) and CPU monitor (20″ Multi-Scan, Apple Computer, Cupertino, CA) allow real-time visualization of the digitized images. IPLab Spectrum software (Scanalytics, Fairfax, VA) orchestrates the entire acquisition process, providing complete automation, as well as postprocessing and analyses of acquired images.

Histology

An osmium tetroxide (OsO_4) paraffin procedure was used to demonstrate fat within harvested *in vivo* disks.[13] Routine staining outlines only "ghost" cells since histological processing with organic solvents and alcohols extract lipid from cells. OsO_4 chemically combines with fat, blackening it in the process. Fat that combines OsO_4 is insoluble in alcohol and xylene and the tissue can be processed for paraffin embedding and counterstained. Small fat droplets and individual cells are well demonstrated via this method, whereas gross amounts of fat are not fixed. After staining with OsO_4, disks are processed for paraffin embedding using standard procedures, except that hemo-D (Fisher) is substituted for xylene. Infiltrated disks are cut and oriented in embedding cassettes as shown in Figure 3. Sections 6 μm thick are cut with a microtome (Leica, Wetzlar, Germany), placed on slides, and coverslipped. Sections are analyzed using brightfield and polarized microscopy. The later allows demarcation of fibrovascular tissue and PLGA by virtue of the fact that oriented collagen fibers in fibrovascular tissue appears high contrast when compared to polymer and void space.

Adipocyte differentiation *in vitro* is routinely monitored using Oil Red O staining for intracellular lipid pools or phase contrast microscopy (lipid appears as phase bright).

Scanning Electron Microscope

Samples of adipocyte-seeded polymer disks, excised epididymal adipose tissue, and disk architecture are treated with a fixative containing 3% glutaraldehyde and 2% paraformaldehyde in 0.1 M cacodylate buffer,

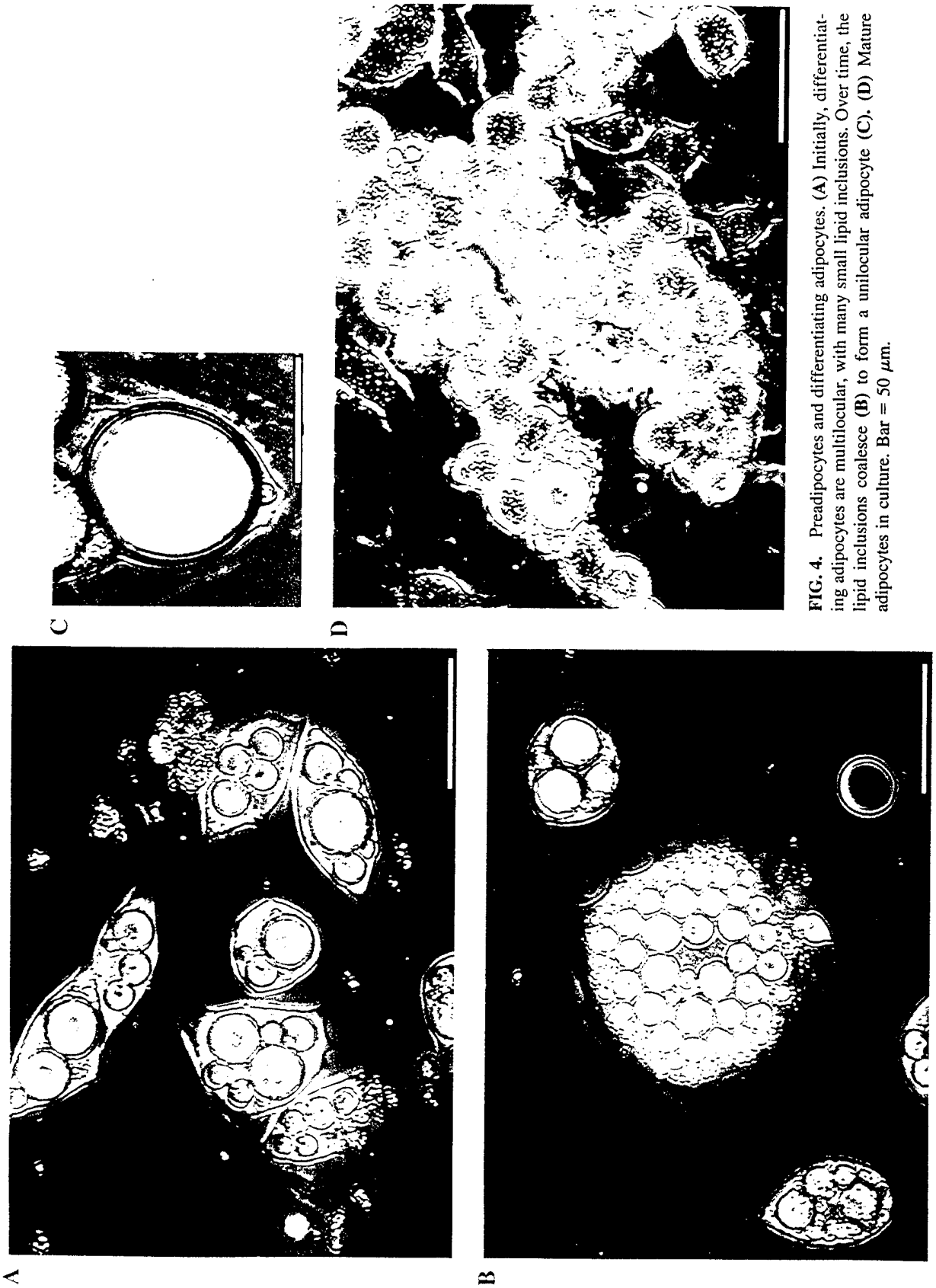

FIG. 4. Preadipocytes and differentiating adipocytes. **(A)** Initially, differentiating adipocytes are multilocular, with many small lipid inclusions. Over time, the lipid inclusions coalesce **(B)** to form a unilocular adipocyte **(C). (D)** Mature adipocytes in culture. Bar = 50 μm.

pH 7.2 for 1 h at room temperature. The samples are rinsed in 0.1 M cacodylate buffer 3× for 5 min/wash and subsequently fixed in cacodylate buffered 1% OsO_4 for 1 h. The samples are then washed in DI water 3× for 5 min/wash and transferred to 1% (aq) thiocarbohydrazide for 10 min. Next, the samples are washed in DI water and transferred to 1% OsO_4 for 10 min and then washed in DI water 3× for 5 min/wash. The samples are dehydrated in a graded series of ethanol, followed by three changes of absolute ethanol, and transferred to 1,1,1,3,3,3-hexamethyldisilazanc (Eastman Kodak Co., Rochester, NY) for 5 min, air dried for 2 h, and vacuum evaporated with Pt/Pd alloy in Balzers MED 010 evaporator (Bal-Tec Products Inc., Middlebury, CT). Samples are examined in a Hitachi S520 SEM operating at an accelerating voltage of 5 kV. Micrographs are recorded on Polaroid 55 P/N film.

RESULTS

In Vitro *Culture and Differentiation*

Primary preadipocytes differentiate from a fibroblast-like spindle morphology with small lipid inclusions, to a multilocular stage with numerous large inclusions, and finally to a state of a single, large lipid inclusion. As lipid accumulates, the plasma membrane shows several micropinocytotic vesicular areas, and the external laminae elaborates. Figure 4 depicts an early preadipocyte with many lipid inclusions, a preadipocyte with a single, large lipid inclusion, and mature adipocytes. Although smaller, the mature adipocytes closely resemble *in vivo* mature adipocytes (Figure 5). It typically required 7–10 days postplating for adipocytes to differentiate into a state typified by Figure 4D. The primary source of lipid for the differentiating adipocytes was 10% FBS. Culturing with 20% and 1% markedly caused an FBS-dependent increase and decrease, respectively, in lipid accumulation (data not shown). Hence, primary preadipocytes were successfully harvested, expanded, and differentiated. In addition, the cells were responsive to changes in lipid loading.

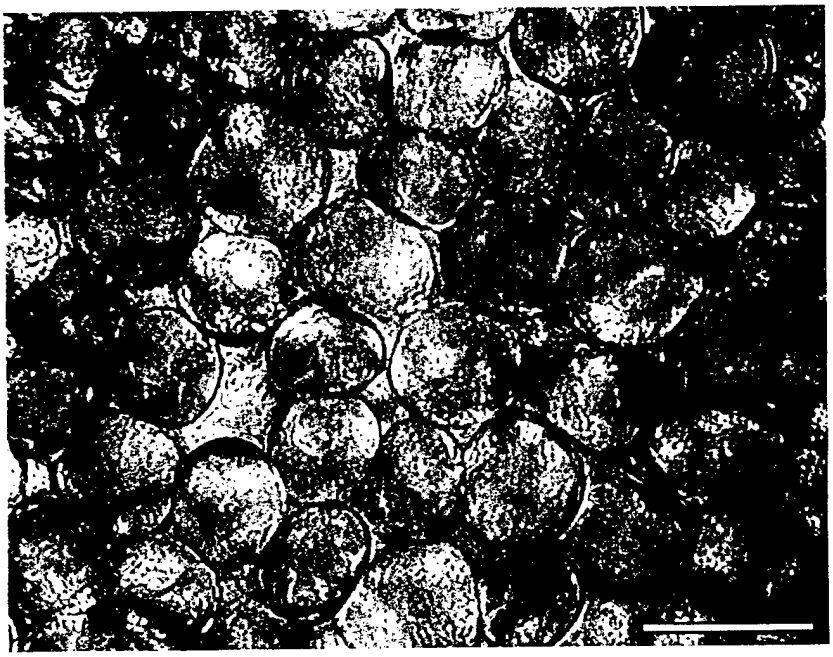

FIG. 5. Mature adipose tissue from rat epididymal fat pads. The tissue is a loose association of lipid filled adipocytes innervated with capillaries and held together with collagen fibers. Bar = 50 μm.

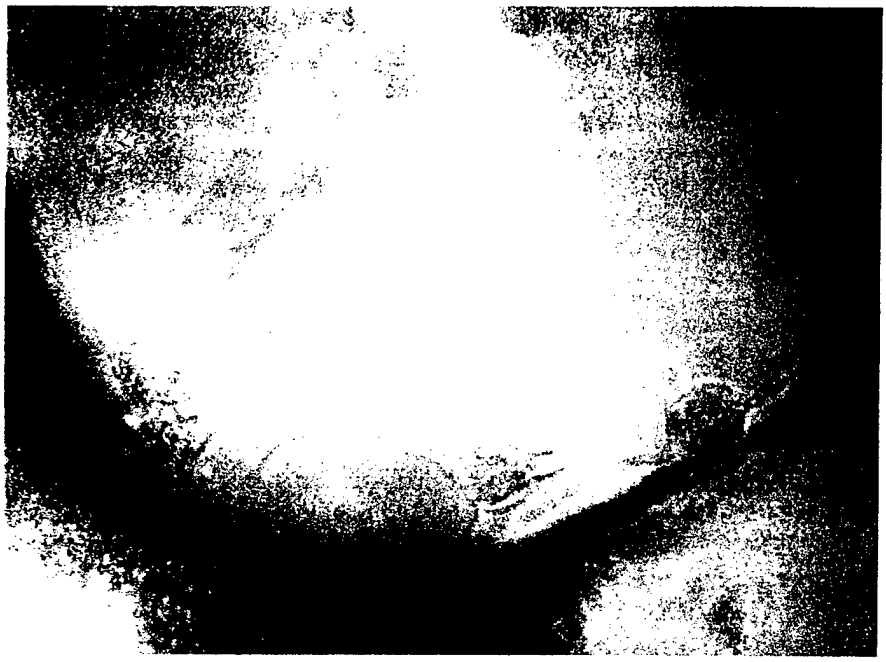

FIG. 6. Differentiated adipocytes observed within the pore of an *in vitro* PLGA disk 11 days postseeding.

In Vitro *Polymer Seeding*

Preadipocytes were cultured, removed from culture flasks, and seeded in PLGA disks to determine the feasibility of cell differentiation within the architecture of a polymer foam. Figure 6 shows differentiated adipocytes adhered to the side of a pore within a PLGA disk 11 days postseeding. This is illustrative of all disks tested. SEMs of preadipocyte-seeded disks reveal that preadipocytes use the pores of the polymer as a scaffold and fill the pores (Figure 7A). It is known that extracellular matrix fibers (predominantly colla-

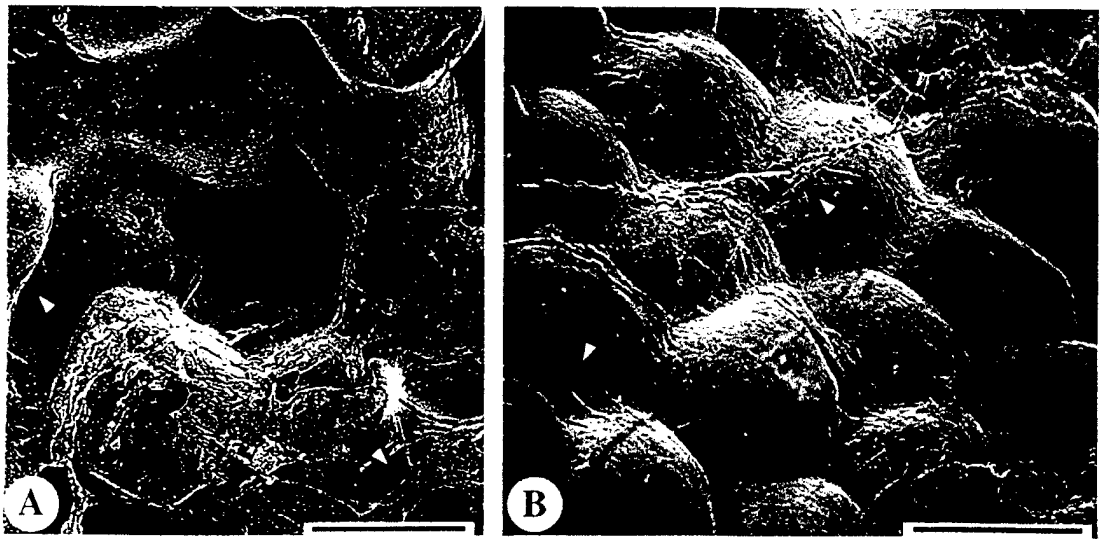

FIG. 7. (**A**) SEM (×100) of preadipocyte-seeded PLGA disks. Adipocytes have adhered to and filled the polymer pores. Arrows denote extracellular matrix fibers and the bar represents 300 μm. (**B**) SEM (×500) of epididymal adipose tissue. Arrows denote extracellular matrix fibers. Bar = 60 μm.

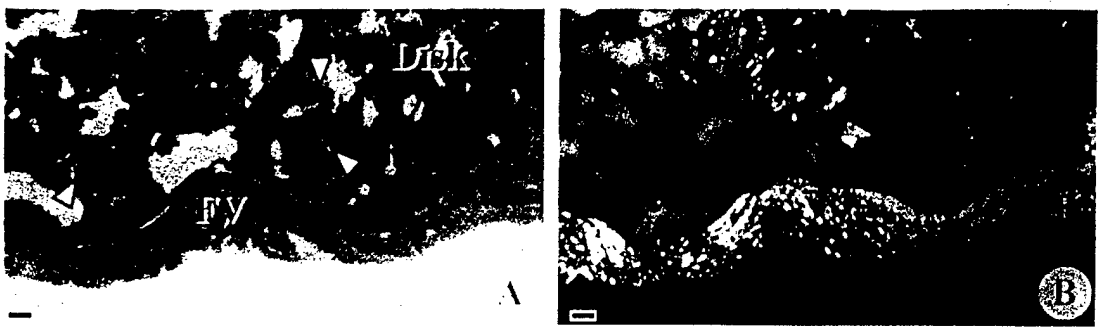

FIG. 8. Use of polarized light **(A)** in conjunction with brightfield optics **(B)** to distinguish polymer boundaries and fibrovascular tissue (FV). Arrows denote black stained adipocytes within the polymer. Bar = 50 μm.

gen I) hold mature adipocytes together.[14] These fibers are present within the preadipocyte-seeded disks (Figure 7A). The adipocyte morphology and extracellular matrix fibers are reminiscent of those observed in epididymal adipose tissue (Figure 7B). Again, as observed in culture flasks, adipocytes in seeded PLGA disks are smaller than those in epididymal adipose tissue.

In Vivo *Polymer Seeding*

Preadipocyte-seeded and acellular disks (control) were implanted in rats for 2 and 5 weeks. The rats were mobile and their range of motion unimpeded by the four disks during the duration of implantation. Harvest of 40 disks revealed a thin layer of fibrovascular tissue around each (mild inflammatory foreign body reaction), but no signs of infection as evidenced by lack of neutrophils. To accurately distinguish between the PLGA disk borders and the fibrovascular tissue under brightfield optics, polarized microscopy was utilized. Collagen organized within the fibrovascular tissue appears brighter than the PLGA under polarized

FIG. 9. OsO_4 staining of acellular (control) PLGA disks harvested after 5 weeks. Adipocytes (black round structures denoted by arrows) are only present within the fibrovascular tissue (FV). The border between the PLGA disk and the fibrovascular tissue is denoted by a heavy black line. Bar = 50 μm.

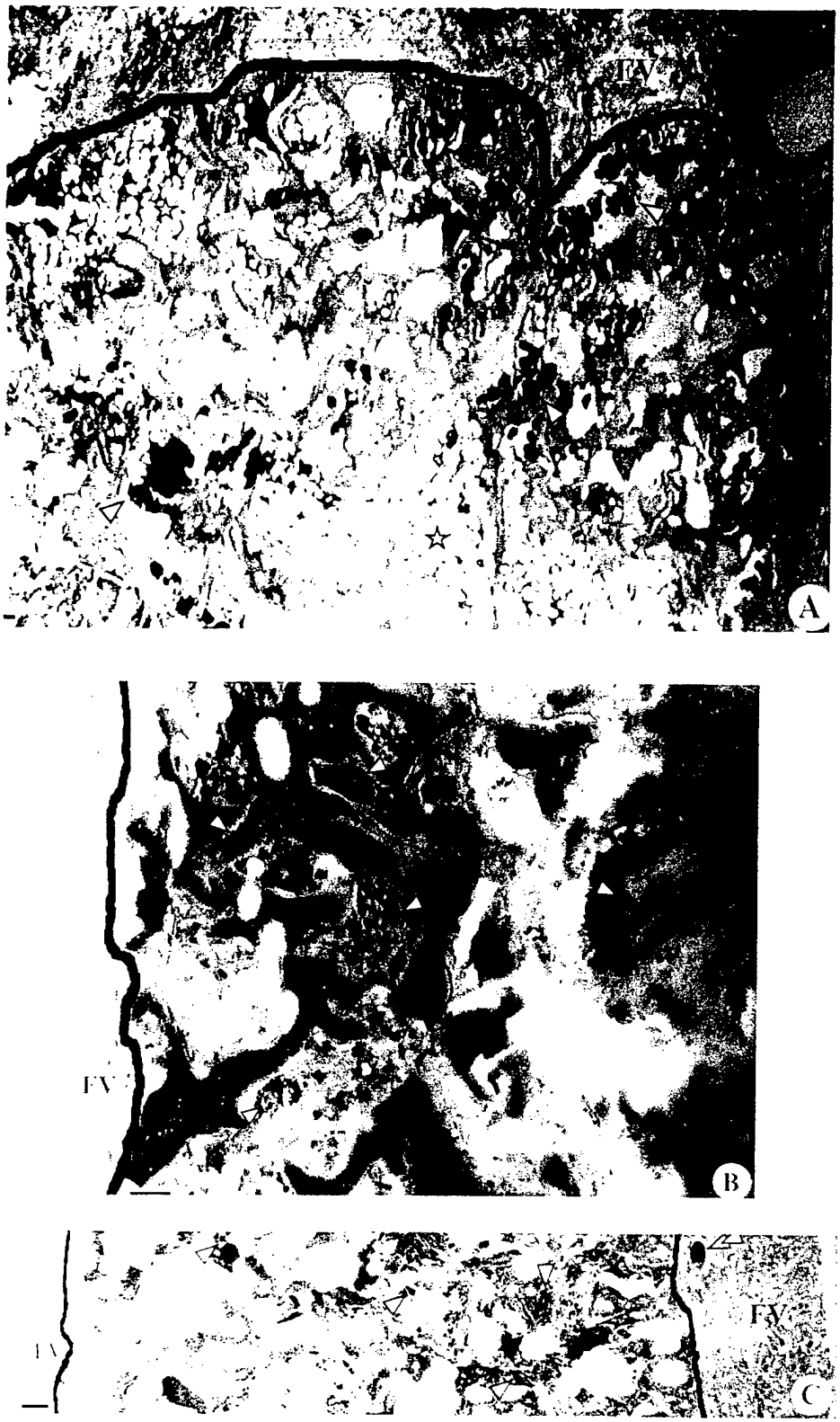

FIG. 10. (A–C) OsO_4 staining of preadipocyte-seeded PLGA disks harvested after 5 weeks. **(A,B)** Face cut sections of disks. **(C)** Cross-section of disk. Adipocytes present within the PLGA disks are denoted by arrows. Stars in A denote gross amounts of fat (unstained), and double arrow in C denotes "host" adipocyte. The border between the PLGA disk and fibrovascular tissue (FV) is denoted by a heavy black line. Bar = 50 μm.

light (Figure 8). No adipocytes were observed within all 10 acellular control disks. Clusters of adipocytes were, however, present in the surrounding fibrovascular tissue as expected (Figure 9). Simple agglomerations of fat cells in connective tissue are known as perivascular fat and differs from adipose tissue by virtue of their blood supply.[7]

All preadipocyte-seeded disks demonstrated the presence of differentiated, mature adipocytes throughout. Figure 10 illustrates representative OsO_4 stained sections of preadipocyte-seeded PLGA disks. In Figure 10A, numerous adipocytes are stained black throughout the entire area of the disk. Since OsO_4 only renders individual adipocytes or lipid droplets insoluble, gross amounts of adipose appear as "ghost" cells (☆ regions in Figure 10A) as it normally appears in conventional histological processing. Importantly, mature adipocytes are present in the center region of the disk as well as the edges. Similarly, Figure 10B illustrates that stained adipocytes are observed within the disk. A cross section through the central region of a PLGA disk is shown in Figure 10C. Differentiated adipocytes are demonstrated throughout the entire thickness of the disk. In addition, Figures 9 and 10 demonstrate that void area is still present after 2 and 5 weeks for preadipocyte expansion (i.e., fibrovascular tissue has not completely filled the disk).

Adipocytes present in the disks do not originate from surrounding fibrovascular tissue, as evidenced by Hoechst labeling of preadipocytes. Labeled preadipocytes were present in seeded disks (Figure 11) and not in acellular disks. Moreover, no labeled preadipocyte was observed to migrate out of the disks into the surrounding perivascular tissue.

FIG. 11. Hoechst labeling of preadipocytes. Fluorescently labeled adipocyte nuclei (blue) overlying a brightfield image of a disk.

DISCUSSION

Previous investigators have isolated preadipocytes from rats[15–19] and humans[20–23] and have demonstrated *in vitro* differentiation. In agreement, primary preadipocytes in this study were initially fusiform and proliferated *in vitro*. Once contact inhibited, the cells accumulated lipid inclusions, became rounder, and acquired an eccentric nucleus. In addition, this study has demonstrated for the first time the attachment, proliferation, and differentiation of preadipocytes within a polymer scaffold, both *in vitro* and *in vivo*. Others have shown that preadipocytes placed *in vivo* (but not in scaffolds) differentiate to mature adipocytes.[19,24] Preseeded PLGA disks implanted in rats demonstrated mature adipocytes throughout the disks at both 2 and 5 weeks.

Admittedly, this study is an initial attempt at adipose tissue engineering and many variables (e.g., cell seeding density, duration of implantation, cell state at implantation time, and polymer architecture) need to be addressed in future experiments. However, there appears to be great potential for using primary preadipocytes as a cell source in cell–seeded polymer scaffolds for soft tissue engineering applications. Preadipocytes have several advantages over mature adipose tissue from a design standpoint. Past investigators have shown that adipose tissue transplants and seeding of mature adipocytes do not result in adequate clinical results or cosmesis for repair of soft tissue defects.[2–7] Mature adipocytes are too fragile,[8,9] do not tolerate hypoxic environments well, and, being fully differentiated, do not proliferate. Because of their undifferentiated state, preadipocytes can be expanded *ex vivo*. Moreover, the initiation of differentiation after expansion is well controlled by simply regulating preadipocyte confluency. Unlike mature adipocytes which are ~90% lipid, preadipocytes are mechanically stable[25] and can be injected into polymers (i.e., they do not "liquefy" during handling or aspiration through a needle). Moreover, preadipocytes are able to tolerate extreme environments (e.g., the hypothesized hypoxic center of the PLGA disks) more easily than fully differentiated cells.

As with research in any new area, the initial data acquired raise many questions. This is compounded greatly in adipose tissue engineering by the fact that the biology of adipose tissue has not been explored in depth, with the exception of how adipocytes relate to obesity. The molecular mechanisms of adipocyte differentiation are just beginning to be explored.[26–30] From a surgical context, adipose grafting, transplantation, and healing remain poorly understood. In addition, very little is known on the basic characterization of fat cells, the features of the cytoplasm and cell membrane receptors, the expression of growth factors, or cell lineage from mesenchymal stem cells. To be sure, the development of clinically translatable adipose tissue equivalents remain challenging and unfulfilled, but results from this study are progressive steps toward realization.

REFERENCES

1. Neuber, G.A. Fettransplantation. Dtsch. Gesellschaft Chir. **22,** 66, 1893.
2. Ersek, R.A. Transplantation of purified autologous fat: a 3-year follow-up disappointing. Plast. Reconstr. Surg. **87,** 219, 1991.
3. Fagrell, D., Eneström, S., Berggren, A., and Kniola, B. Fat cylinder transplantation: an experimental comparative study of three different kinds of fat transplants. Plast. Reconstr. Surg. **98,** 90, 1996.
4. Peer, L.A. The neglected "free fat graft," its behavior and clinical use. Plast. Reconstr. Surg. **11,** 40, 1956.
5. Peer, L.A. Transplantation of fat. In: Converse, J.M., ed. Reconstructive Plastic Surgery: Principles and Procedures in Correction, Reconstruction, and Transplantation. Philadelphia: Saunders, 1977, p. 251.
6. Smahel, J. Failure of adipose tissue to heal in the capsule preformed by a silicone implant. Chir. Plast. **8,** 109, 1985.
7. Smahel, J. Experimental implantation of adipose tissue fragments. Br. J. Plast. Surg. **42,** 207, 1989.
8. Kotonas, T.C., Bucky, L.P., Hurley, C., and May Jr., J.W. The fate of suctioned and surgically removed fat after reimplantation for soft-tissue augmentation: a volumetric and histologic study in the rabbit. Plast. Reconstr. Surg. **91,** 763, 1993.
9. Nguyen, A., Pasyk, K.A., Bouvier, T.N., Hassett, C.A., and Argenta, L.C. Comparative study of survival of autologous adipose tissue taken and transplanted by different techniques. Plast. Reconstr. Surg. **85,** 378, 1990.
10. Mathes, S.J. Clinical Applications for Muscle and Musculocutaneous Flaps. St. Louis, MO: Mosby–Year Book, 1982.

11. American Society of Plastic and Reconstructive Surgeons and Plastic Surgery Education Foundation.
12. Wake, M.C., Patrick, Jr., C.W., and Mikos, A.G. Pore morphology effects on the fibrivascular tissue growth in porous polymer substrates. Cell Transplant. **3,** 339, 1994.
13. Carson, F.L. Osmium tetroxide paraffin procedure. In: Carson, F.L., ed. Histotechnology: A Self-Instructional Text. Chicago: ASCP Press, 1997, p. 153.
14. Wertheimer, E., and Shapiro, B. The physiology of adipose tissue. Physiol. Rev. **28,** 451, 1948.
15. Björntorp, P., Karlsson, M., Pertoft, H., Pettersson, P., Sjöström, L., and Smith, U. Isolation and characterization of cells from rat adipose tissue developing into adipocytes. J. Lipid Res. **19,** 316, 1978.
16. Carraro, R., Lu, Z., Li, Z.H., Johnson, Jr., J.E., and Gregerman, R.I. Adipose tissue islets: tissue culture of a potential source of fat cells in the adult rat. F.A.S.E.B. J. **4,** 201, 1990.
17. Novakofski, J.E. Primary cell culture of adipose tissue. In: Hausman, G.J., and Martin, R.J., eds. Biology of the Adipocyte: Research Approaches. New York: Van Nostrand Reinhold, 1987, pp. 160–197.
18. Van, R.L.R., and Roncari, D.A.K. Isolation of fat cell precursors from adult rat adipose tissue. Cell Tissue Res. **181,** 197, 1977.
19. Van, R.L.R., and Roncari, D.A.K. Complete differentiation *in vivo* of implanted cultured adipocyte precursors from adult rats. Cell Tissue Res. **225,** 557, 1982.
20. Entenmann, G., and Hauner, H. Relationship between replication and differentiation in cultured human adipocyte precursor cells. Am. J. Physiol. **270,** C1011, 1996.
21. Hauner, H., Entenmann, G., Wabitsch, M., et al. Promoting effect of gluctosteroids on the differentiation of human adipocyte precursor cells cultured in a chemically defined medium. J. Clin. Invest. **84,** 1663, 1989.
22. Poznanski, W.J., Waheed, I., and Van, R. Human fat cell precursors: morphologic and metabolic differentiation in culture. Lab. Invest. **29,** 570, 1973.
23. Strutt, B., Khalil, W., and Killinger, D. Growth and differentiation of human adipose stromal cells in culture. In: Jones, G.E., ed. Methods in Molecular Medicine: Human Cell Culture Protocols. Totowa, NJ: Humana Press, 1996, pp. 41–51.
24. Greene, H., and Kehinde, O. Formation of normally differentiated subcutaneous fat pads in an established preadipocyte cell line. J. Cell. Physiol. **101,** 169, 1979.
25. Billings, Jr., E., and May, Jr., J.W. Historical review and present status of free fat graft autotransplantation in plastic and reconstructive surgery. Plast. Reconstr. Surg. **83,** 368, 1989.
26. Brun, R.P., Kim, J.B., Altiok, S., and Spiegelman, B.M. Adipocyte differentiation: a transcriptional regulatory cascade. Curr. Opin. Cell Biol. **8,** 826, 1996.
27. Butterworth, S.C. Molecular events in adipocyte development. Pharmac. Ther. **61,** 399, 1994.
28. Cornelius, P., MacDougald, O.A., and Lane, M.D. Regulation of adipocyte development. Annu. Rev. Nutr. **14,** 99, 1994.
29. MacDougald, O.A., and Lane, M.D. Transcriptional regulation of gene expression during adipocyte differentiation. Annu. Rev. Biochem. **64,** 345, 1995.
30. Smas, C.M., and Sul, H.S. Control of adipocyte differentiation. Biochem. J. **309,** 697, 1995.

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LONG-TERM IMPLANTATION OF PREADIPOCYTE SEEDED PLGA SCAFFOLDS

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ABSTRACT

Studies were performed in a long-term effort to develop clinically translatable, tissue engineered adipose constructs for reconstructive, correctional, and cosmetic indications. Rat preadipocytes were harvested, isolated, expanded *ex vivo*, and seeded within PLGA scaffolds. Preadipocyte-seeded and acellular (control) scaffolds were implanted for times ranging from 1 to 12 months. Explanted scaffolds were stained with osmium tetroxide, processed, and counterstained using H&E. Quantitative histomorphometric analysis was performed on all tissue sections to determine the amount of adipose tissue formed. Analyses revealed maximum adipose formation at 2 months, followed by a decrease at 3 months, and complete absence of adipose and PLGA at 5 through 12 months. These results extend a previous short-term study (*Tissue Engineering* 5:134,1999) and demonstrate that adipose tissue can be formed *in vivo* using tissue engineering strategies. However, the long-term maintenance of adipose tissue remains elusive.

INTRODUCTION

The application of tissue engineering to the development of adipose tissue constructs has captured the interests of numerous investigators over the past two years. [1-8] This is due in part to the realization that there are many reconstructive, correctional, and cosmetic indications for patient-specific adipose constructs. [4] In a previous qualitative study, we demonstrated adipose tissue formation within preadipocyte (PA)-seeded PLGA scaffolds implanted for 2 and 5 weeks. [3] Many questions were raised at the conclusion of this initial study. One such question is whether adipose tissue that forms within PLGA scaffolds remains over long periods of time and whether it remains after its supporting polymer scaffold entirely degrades. This question merits consideration based on the longstanding observation that transplanted mature fat resorbs over time.

This present study is a continuation and elaboration of its former. Specifically, PAs are seeded within PLGA scaffolds and implanted for times ranging from 1 to 12 months. Adipose formation is quantitatively assessed by coupling histology with microscopy and image analysis.

MATERIALS & METHODS

Adipose Harvest & In Vivo Culture

The methods for harvesting and culturing the PAs were previously described. [3,9] Briefly, PAs were isolated from epididymal fat pads of male, 250 g, 70-80 day old Lewis rats (Harlan) via enzymatic digestion. Rats were euthanized with CO_2 asphyxiation and the shaved harvest site was scrubbed with Betadyne followed by alcohol wash. Within 5 minutes of death, epididymal adipose tissue was aseptically harvested and placed in 4°C saline solution supplemented with 500 U/mL penicillin and 500 μg/mL streptomycin (Gibco). Using a dissecting microscope, connective tissues and tissue containing blood vessels were resected from the fat. This minimizes fibroblast contamination of *ex vivo* cultures. Harvested tissue was finely minced with a scalpel and enzymatically digested in Ca^{+2}/Mg^{+2}-free saline supplemented with 2% (w/v) type I collagenase (Sigma Chemical Co.) and 5% (w/v) bovine serum albumin (BSA) for 20 minutes at 37°C on a shaker. For 4 fat pads, 5 mL of dissociation medium is required. The digested tissue was filtered through a 250 μm mesh followed by a 90 μm nylon mesh to separate undigested debris and capillary fragments from PAs. The filtered cell suspension was centrifuged and the resulting pellet of PAs was then plated at 10^4 cells/cm^2 onto plastic culture flasks. PAs were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 μg/mL streptomycin. During cell expansion, the PAs were passed prior to confluency since contact inhibition initiates adipocyte differentiation and ceases PA proliferation. [10-15] The 1° passage yields approximately $1.5x10^6$ PAs/fat pad.

Polymer Fabrication & Seeding

PLGA is employed as a model polymer. PLGA foam fabrication and seeding were conducted as previously described. [3] Fabrication of 2.5 mm thick, 12 mm diameter, and 90% porosity polymer disks were prepared by a particulate-leaching technique. Briefly, 5 g solid 75:25 PLGA (Birmingham Polymers Inc.) polymer were dissolved in 80 mL dichloromethane (Fisher Scientific) to form a solution. Sieved NaCl crystals (Fisher) at a NaCl:PLGA weight fraction of 1:9 were evenly dispersed over a 150 mm Pyrex petri dish (Fisher) with a Teflon lining (Cole-Parmer Instrument Co.). The PLGA/dichloromethane solution was then gently poured over the NaCl crystals. Sieved NaCl crystal size distribution was measured with quantitative microscopy and found to be 135-633 μm. Dichloromethane was evaporated under vacuum, leaving a polymer/NaCl composite 2.5 mm thick. The composite was removed from the Teflon-lined petri dish and 12 mm diameter disks were cut using a plug cutter and drill press. The NaCl crystals were then leached from the composite disks by immersion in 800 mL of DI water for 48 hours (water changed every 8 hours) to yield porous disks. Disks were lyophilized and stored in a vacuum dessicator until use.

Prior to seeding, the test materials were prewetted and sterilized with absolute ethanol for 30 minutes followed by two sterile saline washes at 20 minutes/wash and a DMEM wash for 20 minutes. A 20 μL suspension of PAs (10^5 cells/mL) was injected onto each disk under sterile conditions. Prewetting permits the cell suspension to readily flow throughout the materials. Following 3 hours for cell attachment, 24-well culture plates containing one disk/well were filled with 1.5 mL of medium/well. Before transporting the cell-seeded constructs to the operating room, DMEM media was removed and replaced with complete L-15 media, and the constructs were placed in a mobile 37°C warmer. The use of L-15 precludes the need for 5% CO_2 for pH control.

In Vivo Implantation

Seeded disks were implanted on the back musculatureof Lewis rats under anesthesia (0.2 mL/100 gbw intramuscular injection of premixed solution composed of 64 mg/mL ketamine HCl, 3.6 mg/mL xylazine, and 0.07 mg/mL atropine sulfate). [3,9] An isogenic strain is required to avoid an immune response to seeded PAs. The University of Texas M.D. Anderson Cancer Center Animal Care and Use Committee has approved the implantation of PA seeded disks. After shaving the back, two longitudinal incisions (~2 cm each) were made through the skin of the dorsal midline. Individual "pockets" for each disk were prepared between the cutaneous trunci and back muscles of both flanks by careful dissection. Disks were inserted into each pocket and sutured in place with 5-0 suture (Ethicon), as shown in Figure 1A. Two disks were placed on each side of the incision (4 disks/rat) and the incisions closed with 4-0 suture (Ethicon) (Figure 1B). Animals were housed individually and fed standard rat chow. The disks were left *in vivo* 1, 2, 3, 5, 7, 9, and 12 months. After the elapsed time, the rats were euthanized with CO_2 and the disks harvested. Immediately after harvest, the disks were placed in 10% neutral buffered formalin (Fisher) for future histology.

For this study, a total of 42 rats were used at 6 rats/time period. Each rat was implanted with 4 disks. Two disks were seeded with PAs and two disks were implanted without seeding to serve as acellular controls. A total of 84 seeded and 84 acellular disks were used in this study.

Histology

An osmium tetroxide (OsO_4) paraffin procedure was used to demonstrate fat within half of the harvested *in vivo* disks. [3] The remaining half of the disks were frozen for storage and future analyses. Routine staining outlines only "ghost" cells since histological processing with organic solvents and alcohols extract lipid from cells. OsO_4 chemically combines with fat, blackening it in the process. Fat that combines OsO_4 is insoluble in alcohol and xylene and the

tissue can be processed for paraffin embedding and counterstained. After staining with OsO_4, disks were processed for paraffin embedding using standard procedures, except that Histosolve (Shandon) was substituted for xylene. Sections 4 μm thick were cut on a microtome (Leica), placed on slides, counterstained with H&E, and coverslipped to view fibrovascular tissue. Sections were analyzed using brightfield microscopy.

Determination of Adipose Tissue

High magnification images of histology slides were acquired using an inverted microscope (Olympus), color CCD camera (Olympus), computer-controlled XYZ stage (Ludl Electronic Products), IPLab software (Scanalytics), and a PowerPC (Apple). Images were acquired at a resolution of 0.61 pixels/μm using a 20x, 0.40 NA objective (Olympus). A depiction of the image analysis strategy is shown in Figure 2. Three full thickness images were acquired automatically from each slide: at the center, ~3 mm right of the center, and ~3 mm left of the center. Image acquisition consisted of digitally tiling eight 640 x 480 pixel images for each full thickness image. Hence, each image was 640 x 3,840 pixels or 390 μm x 2,342 μm (Figures 3A and 3D). Next, using image segmentation, the user selected areas of adipose tissue formation (*i.e.*, OsO_4-stained regions) consisting of mature adipocytes or lipid-filled differentiating PAs (Figure 3B and 3E). The total scaffold area was determined (Figure 3C). Data are presented as percent of adipose tissue, defined as:

$$\% \text{ Adipose Formation} = 100 \bullet \left(\frac{\sum \text{Left, Center, Right Adipose Area}}{\sum \text{Left, Center, Right Scaffold Area}} \right)$$

Data from center, left, and right full thickness images were pooled, resulting in one value for each slide rather than three.

RESULTS

There were no complications due to surgery or postoperative recovery from anesthesia. Scaffold harvest was unremarkable with no hematomas or seromas. Scaffolds remained intact and appeared to be well-vascularized based on gross inspection (Figure 1B). As shown in Figure 4, the PLGA disks were sutured in direct apposition to muscle, with no void space or fascia present. Hence, barriers to scaffold revascularization were minimized.

Figure 5 demonstrates the percent adipose formation within the acellular and PA seeded PLGA scaffolds. For months 1 through 3, PA-seeded scaffolds demonstrated statistically more adipose tissue than their acellular controls. Adipose tissue formation appears to peak at 10.7% at 2 months followed by a decrease. PLGA scaffolds were entirely degraded by 5 months. No PLGA scaffold or fat tissue was present in rats harvested 5 months to 12 months. The absence of PLGA at ≥5 months is in keeping with the 75:25 copolymer's approximate degradation kinetics of 4-5 months. Exact degradation kinetics is dependent on scaffold geometry, porosity, and molecular weight.

A thin layer of adipose tissue was observed within the fibrovascular tissue (i.e., foreign body capsule) around, but outside, both acellular and PA-seeded disks (Figure 6). The thin layer of adipose tissue was highly vascular and is presumably formed from resident PAs recruited to the foreign body capsule. This observation was noted in the former short-term study [3] and with proprietary cell-seeded and acellular polymers from Johnson & Johnson Corporate Biomaterials

Center (data not shown). There was no difference in the layer characteristics at implantation times of 1, 2, and 3 months or between acellular and PA-seeded disks.

Histologically, the amount of all tissue (fibrovascular and adipose combined) decreased between 1 and 3 months as the PLGA degraded (Figure 7). Without polymer support, the tissue at 3 months formed threads of connecting tissue (Figures 4C, 4D). The amount of macrophage infiltration qualitatively decreased with time as well between 1 and 3 months (Figure 8). Macrophage presence was localized to polymer-tissue interfaces.

DISCUSSION

This long-term study expanded the results observed with a previous short-term study that demonstrated adipose formation within PA-seeded PLGA scaffolds after 2 and 5 weeks of transplantation. [3] This study addresses the issue of long-term maintenance of adipose formation within a model polymer-cell system. Based on the animal model utilized, one concludes that PA-seeded PLGA scaffolds permit increasing adipose formation that peaks at approximately 2 months and decreases dramatically thereafter. It is encouraging to demonstrate viable adipose tissue formation within PA-seeded PLGA scaffolds for up to 2 months. However, the exact microenvironment required for long-term maintenance remains elusive.

There are several possible explanations for the resorption observed in this long-term study. One explanation may be related to the anatomical site of transplantation. Epididymal PAs were seeded within PLGA scaffolds and transplanted to the dorsal subcutaneous region of the rat between two muscle beds, namely the panniculus carnosus (a.k.a., cutaneous trunci) muscle and skeletal muscles of the back. The thin layer of fascia separating the muscle beds was removed prior to construct implantation. Rats typically possess very little subcutaneous adipose

tissue. Hence, the microenvironment present in the current model may not support long-term maintenance of adipose tissue. This may be a limitation of the animal model employed. Research is currently being conducted on characterizing a porcine model which has the potential of being a more amenable subcutaneous adipose model. Further, adipose tissue physiology (i.e., rate of replication, capacity for differentiation) is dependent on anatomical location. [16-19] That is, there are site-specific characteristics intrinsic to the PAs that result in regional variations in properties of adipose tissue. Thus, a subcutaneous microenvironment may not optimally support epididymal PAs.

Another explanation for the resorption observed may be the degree of vascularization. It has been known for decades that adequate vascular supply is essential for generation and maintenance of adipose tissue. ECM components secreted by microvascular endothelial cells have been shown to directly stimulate PA differentiation and replication. [20] When avascular constructs are transplanted *in vivo*, the angiogenesis response observed is that of a wound healing cascade. During the late stages of wound healing, there is significant vascular remodeling and involution. The regression of the neovascularization would intuitively result in a concomitant decrease in adipose volume. Moreover, as the amount of PLGA scaffold decreases with time, the influx of macrophages will decrease, as was observed in this study (Figure 8). Likewise, the level of macrophage-secreted angiogenic factors will decrease resulting in regression of neovascularization. The exact level of vascularization was not determined in this study due to the fact that reagents required for CD31 staining of endothelial cells [21] dissolve the PLGA scaffold. However, gross examination of explants and qualitative examination of H&E stained sections demonstrate numerous vessels at all time periods.

Long-term maintenance of adipose formation may require a specific, continued support structure. Anatomically, adipose tissue is held together by a network of ECMs (primarily collagen I) and is typically located in a defined anatomical space (e.g., the breast skin envelope for mammary adipose or between dermis and muscle for subcutaneous adipose). Although exact PLGA degradation was not assessed in this study, it is intriguing to note that the absence of adipose tissue corresponded to the absence of PLGA scaffold at time points ≥5 months. Further, at 3 months, the tissue within the disks formed thin interconnecting threads (Figures 7C, 7D, and 7C). One could speculate that the tissue was losing its support structure by 3 months. The use of 75:25 PLGA as a model polymer may limit the current model due to its relatively short degradation time. Using a biomaterial that degrades more slowly may allow adipose tissue to become more mature and maintain its presence longer than 2-3 months.

Finally, the resorption may be related to the lack of a continued, specific microenvironment. The microenvironment of PA regenerative proliferation and differentiation needs to be maintained at the site of transplantation. Several investigators have demonstrated the recruitment of endogenous PAs and *de novo* adipose formation when the unique microenvironment consisting of Matrigel and bFGF are created in small animal models. [7,22,23] In this present study and others, we have observed formation of *de novo* adipose tissue within the fibrovascular capsule surrounding implanted acellular and PA-seeded biodegradable polymers (Figure 6). The adipose tissue forms a thin layer parallel to the polymer surface and is surrounded by copious blood vessels (Figure 6). Although speculative, endogenous PAs may recruit to the highly vascular regions and/or be attracted by factors released by early macrophage invasion.

The apparent resorption of adipose tissue with extended periods is not a new problem. Numerous strategies have been attempted to prevent adipose resorption following grafting, including using small diameter grafts and growth factors. The results of this study illustrate that the long-term maintenance of engineered adipose tissue is not a trivial task. In addition, results of this study suggest that a different combination of cell source, biomaterial, and animal model may lead to more mature adipose tissue for the study of long-term fat maintenance.

ACKNOWLEDGEMENTS

We thank the following individuals from University of Texas' Laboratory of Reparative Biology & Bioengineering for their technical assistance with the *in vivo* study: Kristen Dempsey, Cynthia Frye, Gary Klaussen, Shannon Scott, and May Wu. This study was supported in part by a U.S. Army grant (DAMD17-99-1-9268), Cancer Fighter's of Houston grant, and a National Institutes of Health grant (CA16672).

REFERENCES

1. Lee, K.Y., Halberstadt, C.R., Holder, W.D., Mooney, D.J. Breast reconstruction. In: Lanza, R.P., Langer, R., Vacanti, J., eds. Principles of Tissue Engineering. San Diego: Academic Press, 2000, pp. 409-423.

2. Patrick Jr., C.W., Chauvin, P.B., Robb, G.L. Tissue engineered adipose. In: Patrick Jr., C.W., Mikos, A.G., McIntire, L.V., eds. Frontiers in Tissue Engineering. Oxford: Elsevier Science, 1998, pp. 369-382.

3. Patrick Jr., C.W., Chauvin, P.B., Reece, G.P. Preadipocyte seeded PLGA scaffolds for adipose tissue engineering. Tissue Eng **5**, 139-151, 1999.

4. Patrick Jr., C.W. Adipose tissue engineering: The future of breast and soft tissue reconstruction following tumor resection. Sem Surg Oncol **19**, 302-311, 2000.

5. Kral, J.G., Crandall, D.L. Development of a human adipocyte synthetic polymer scaffold. Plast Reconstr Surg **104**, 1732-1738, 1999.

6. Yuksel, E., Weinfeld, A.B., Cleek, R., Waugh, J.M., Jensen, J., Boutros, S., Shenaq, S.M., Spira, M. *De novo* adipose tissue generation through long-term, local delivery of insulin and insulin-like growth factor-1 by PLGA/PEG microspheres in an *in vivo* rat model: A novel concept and capability. Plast Reconstr Surg **105**, 1721-1729, 2000.

7. Beahm, E., Wu, L., Walton, R.L. Lipogenesis in a vascularized engineered construct. American Society of Reconstructive Microsurgeons. San Diego, CA; 2000

8. Patrick Jr., C.W. Tissue engineering strategies for soft tissue repair. Anatomical Record in press.

9. Patrick Jr., C.W., Wu, X., C.Johnston, Reece, G.P. Epithelial cell culture-Breast. In: Atala, A., Lanza, R., eds. Methods in Tissue Engineering: Academic Press, in press, pp.

10. Ailhaud, G., Amri, E.Z., Bardon, S., Barcellini-Couget, S., Bertrand, B., Catalioto, R.-M., Dani, C., Djian, P., Doglio, A., Forest, C., Gaillard, D., Grimaldi, P., Negrel, R., Vannier, C., Vassaux, G. The adipocyte: relationships between proliferation and adipose cell differentiation. Am Rev Respir Dis **142**, S57-S59, 1990.

11. Butterwith, S.C. Molecular events in adipocyte development. Pharmaceutical Therapeutics **61**, 399-411, 1994.

12. Cornelius, P., MacDougald, O.A., Lane, M.D. Regulation of adipocyte development. Annual Review of Nutrition **14**, 99-129, 1994.

13. MacDougald, O.A., Lane, M.D. Transcriptional regulation of gene expression during adipocyte differentiation. Annual Review of Biochemistry **64**, 345-373, 1995.

14. Ntambi, J.M., Kim, Y-C. Adipocyte differentiation and gene expression. J Nutr **130**, 3122S-3126S, 2000.

15. Smas, C.M., SookSul, H.S. Control of adipocyte differentiation. Biochem. J. **309**, 697-710, 1995.

16. Djian, P., Roncari, D.A.K., Hollenberg, C.H. Influence of anatomic site and age on the replication and differentiation of rat adipocyte precursors in culture. J Clin Invest **72**, 1200-1208, 1983.

17. Hauner, H., Entenmann, G. Regional variation of adipose differentiation in cultured stromal-vascular cells from the abdominal and femoral adipose tissue of obese women. Int J Obesity **15**, 121-126, 1991.

18. Kirkland, J.L., Hollenberg, C.H., Kindler, S., Gillon, W.S. Effects of age and anatomic site on preadipocyte number in rat fat depots. J Gerontology **49**, B31-B35, 1994.

19. Kirkland, J.L., Hollenberg, C.H., Gillon, W.S. Effects of fat depot site on differentiation-dependent gene expression in rat preadipocytes. International Journal of Obesity Related Metabolic Disorders **20 Suppl 3**, S102-S107, 1996.

20. Varzaneh, F.E., Shillabeer, G., Wong, K.L., Lau, D.C.W. Extracellular matrix components secreted by microvascular endothelia cells stimulate preadipocyte differentiation in vitro. Metabolism **43**, 906-912, 1994.

21. King, T.W., Johnston, C., Patrick Jr., C.W. Quantification of vascular density using a semi-automated technique for immuno-stained specimens. Analyt Quant Cytol Histol in press.

22. Kawaguchi, N., Toriyama, K., Nicodemou-Lena, E., Inou, K., Torii, S., Kitagawa, Y. De novo adipogenesis in mice at the site of injection of basement membrane and basic fibroblast growth factor. Cell Biology **95**, 1062-1066, 1998.

23. Tabata, Y., Miyao, M., Inamoto, T., Ishii, T., Hirano, Y., Yamaoki, Y., Ikada, Y. De novo formation of adipose tissue by controlled release of basic fibroblast growth factor. Tissue Engineering **6**, 279-289, 2000.

FIGURES

FIG. 1. (A) Example of preadipocyte-seeded disk transplantation (B) Example of disk harvest (1 month).

FIG. 2. Illustration depicting image analysis strategy. Three full thickness images were acquired for each histological section of seeded/control PLGA disks-a center image and left/right images 3 mm from the center. Each full thickness image was constructed by tiling eight individual 640 x 480 pixel images.

FIG. 3. Illustration depicting routing of full thickness image tiling and segmentation. (A) and (D) illustrate osmium tetroxide-labeled adipose tissue. (B) and (E) illustrate segmented adipose tissue area (yellow) of (A) and (E), respectively. (C) Illustrates segmented disk area (green) of (A). (D) and (E) are higher magnification images of areas denoted by red box in (A) and (B), respectively.

FIG. 4. Representative histology (H&E) depicting the interface between PLGA disks and skeletal muscle beds of the rat. There is no void space or fascia present at the interface, only a thin layer of fibrovascular tissue (FVT). The entire PLGA disk thickness is not shown. Magnification is 100x.

FIG. 5. Percent adipose tissue formation in acellular and preadipocyte (PA)- seeded scaffolds vs. implantation time. Data are mean±SEM of n=6. * denotes a statistical difference in adipose

tissue presence between acellular and PA seeded scaffolds ($p \leq 0.05$). § denotes rats with entirely resorbed PLGA scaffolds and no adipose tissue.

FIG. 6. Representative histology (OsO_4 and H&E) of thin layer of vascular adipose tissue observed outside acellular (A) and PA-seeded (B) disks. There were no differences in perivascular adipose formation between acellular and PA-seeded disks. Magnification is 100x.

FIG. 7. Representative images (OsO_4 and H&E) depicting extent of tissue presence at 1 month (A), 2 months (B), and 3 months (C, D). (D) Higher magnification of area denoted in (C). Magnification is 40x for (A)-(C) and 200x for (D).

FIG. 8. Representative images (OsO_4 and H&E) depicting extent of macrophage infiltration at 1 month (A), 2 months (B), and 3 months (C). FB denotes fibroblasts. M denotes macrophages. P denotes polymer. Magnification is 400x.

Figure 1

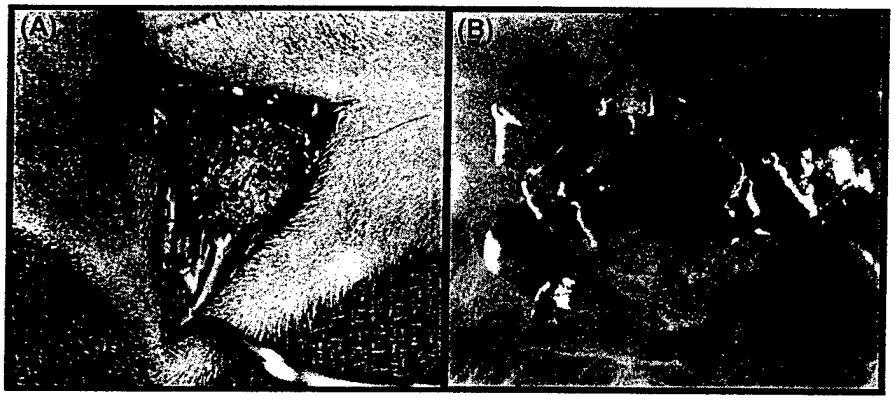
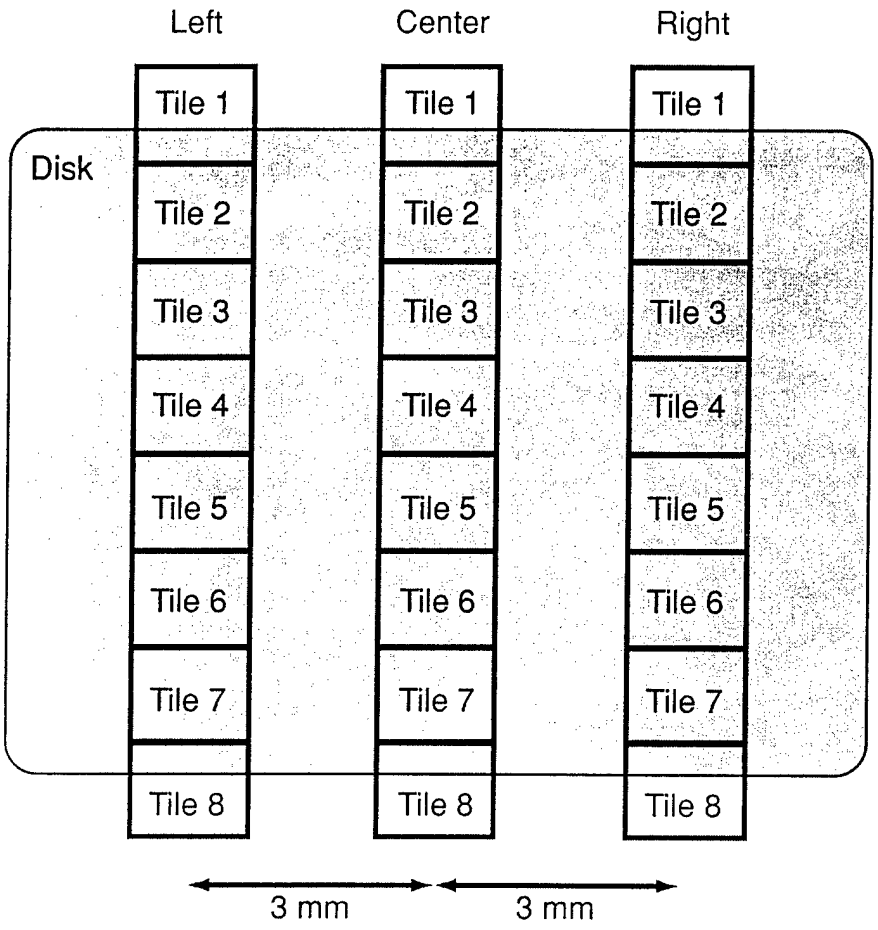


Figure 2


Left
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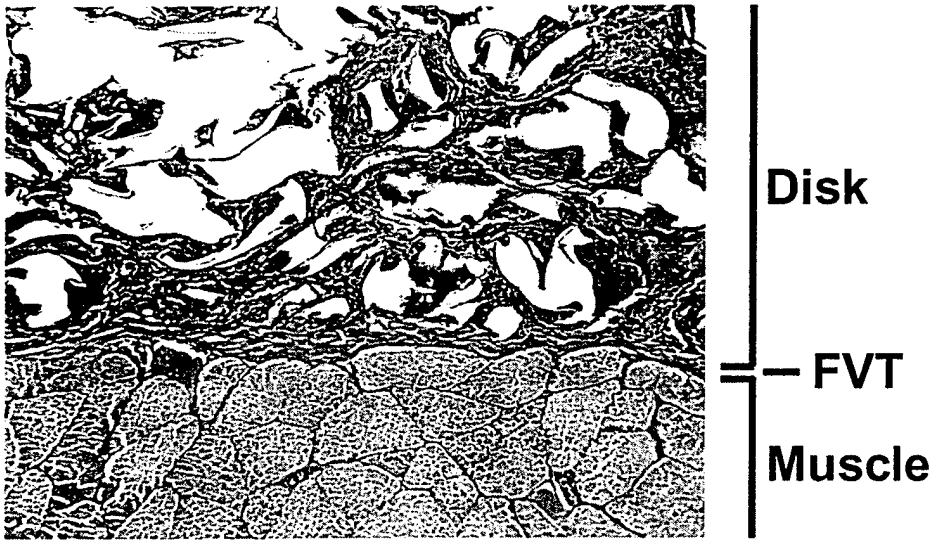
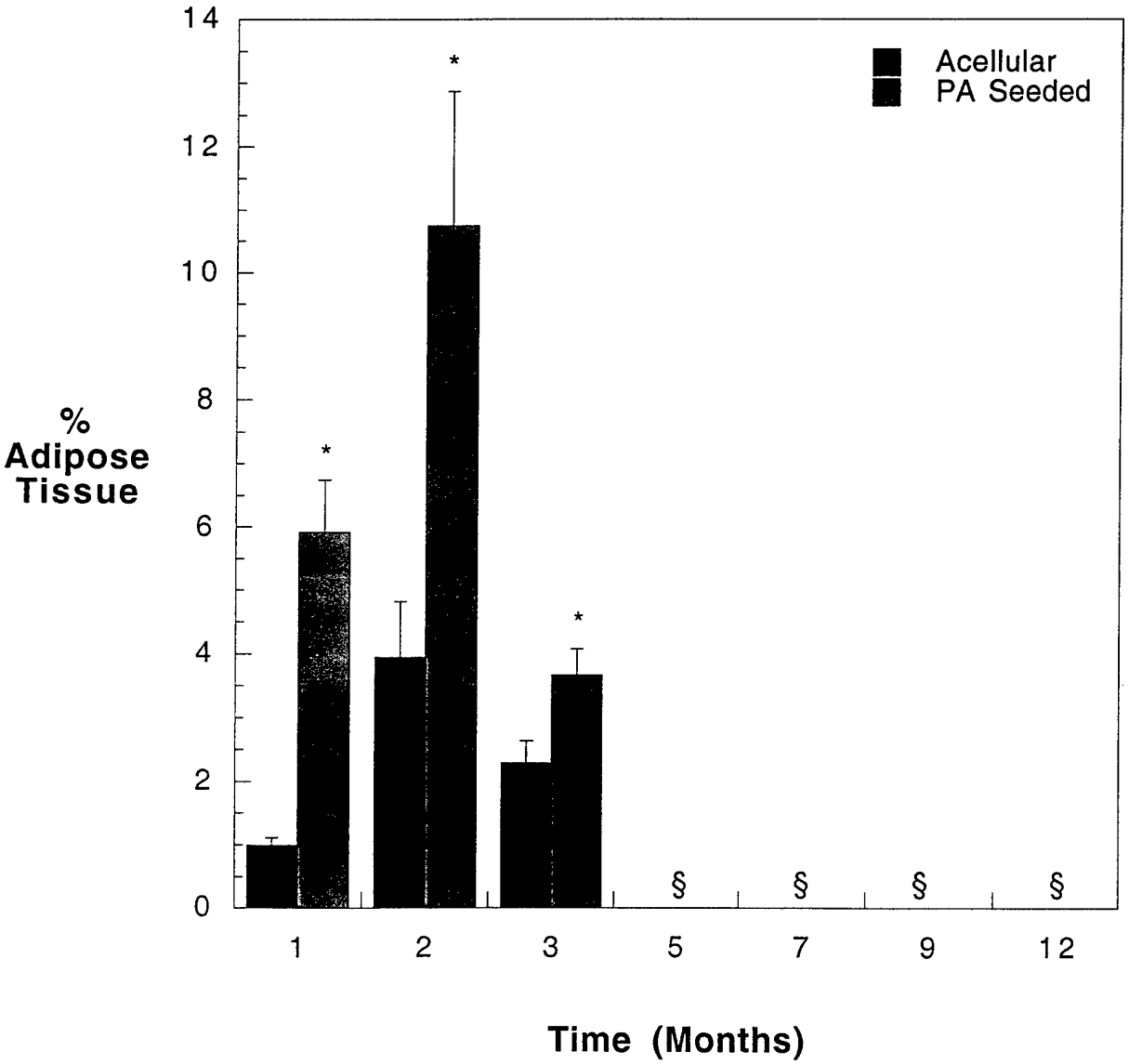
(A)
(B)
(C)


Disk
FVT
Muscle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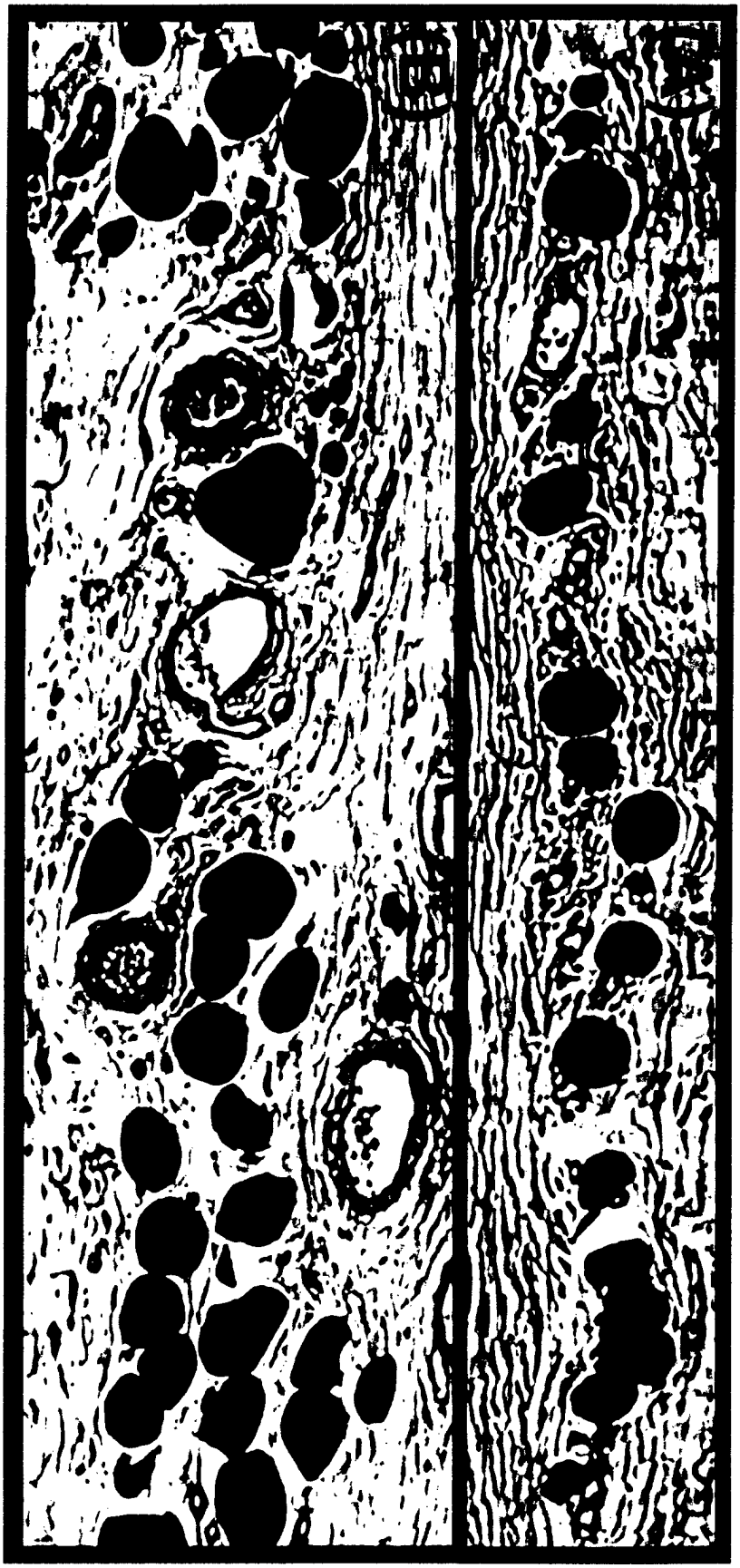

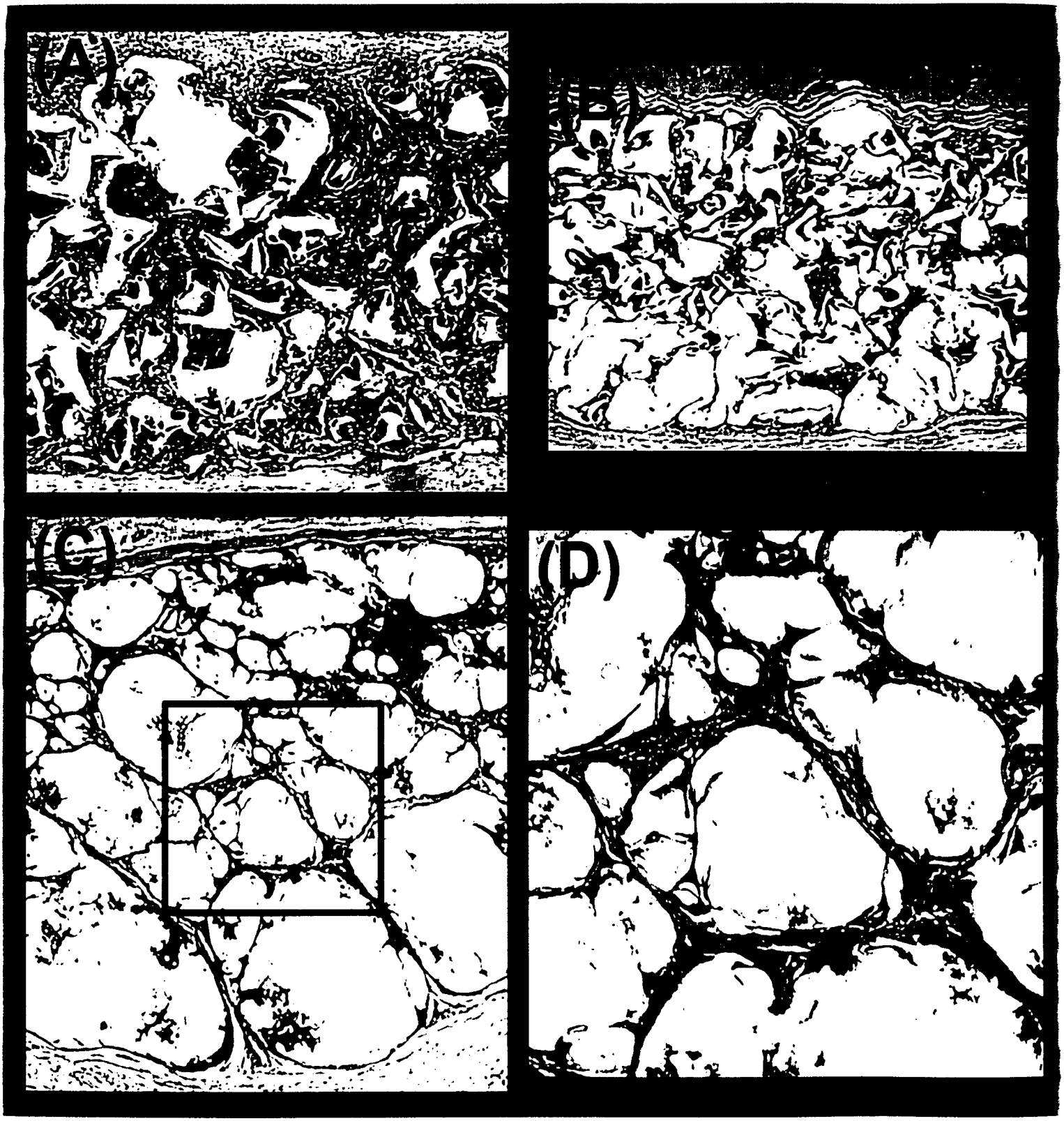
(A)
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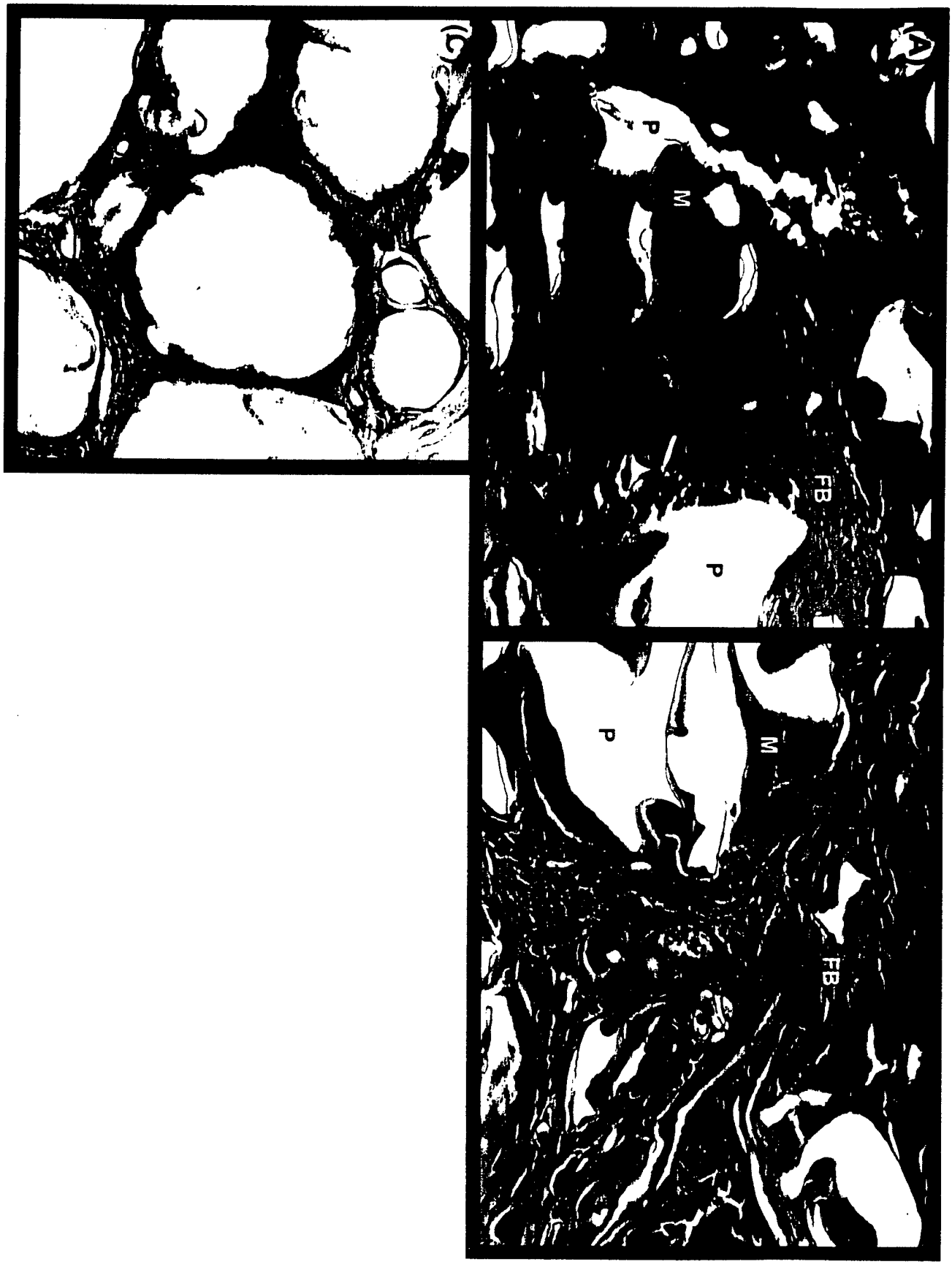
Figure 8



Galley Proof

Methods in Tissue Engineering
Academic Press
(in press)

CHAPTER 10

EPITHELIAL CELL CULTURE: BREAST

Charles W. Patrick Jr., Xuemei Wu, Carol Johnston, and Greg P. Reece

INTRODUCTION

A breast is composed of many various tissue types, including blood vessels, adipose tissue, lymphatic vessels, connective tissue, and mammary glands. This chapter focuses on one tissue type, namely, adipose tissue. With advances in the molecular and cellular biology of adipose tissue, largely from the obesity and diabetes fields, and the maturation of tissue engineering, there has been a resurgence in the potential use of preadipocytes (adipogenic progenitor cells) in clinical strategies. Strategies under current investigation include soft tissue augmentation [1–4] and development of *de novo* breast [1,5] (also see Chapter 78 of this text). Preadipocytes are extremely attractive candidates for tissue engineering. Adipose tissue is uniquely expendable and abundant among most humans. Moreover, preadipocytes can easily be obtained from biopsied or excised fat and from minimally invasive liposuction aspirates. Unlike mature adipocytes, preadipocytes can withstand the mechanical trauma of aspiration and injection, as well as periods of ischemia. In addition, preadipocytes can be expanded into large numbers *ex vivo*, and the biological mechanisms dictating preadipocyte-to-adipocyte conversion are known and can be controlled [6–11].

← ok? (Breast Reconstr. Reid et al.)

This chapter describes the harvest, isolation, and *in vitro* culture of rat preadipocytes, as well as polymer seeding and histology of preadipocytes. Epididymal fat pads are utilized as the preadipocyte source, although other adipose tissue stores may be used. Implant integration, histogenesis, and the use of preadipocyte cell lines [12–19] are not discussed. Moreover, the influence of rat age and anatomic site on preadipocyte characteristics is not discussed [20–22]. All techniques can be scaled appropriately for other animal models and human tissue [23–31]. Moreover, the techniques utilize standard laboratory or easily obtainable equipment and supplies.

HARVEST AND ISOLATION OF PREADIPOCYTES

The following steps are required for harvest of epididymal fat pads from rats and isolation of preadipocytes from the fat pads. Steps 1–12 are conducted in an animal necroscopy facility, and steps 13–22 are conducted in a tissue culture facility. The presented procedures are in accordance with the guidelines of the American Association for Accreditation of Laboratory Animal Care (AAALAC) and the National Institutes of Health (NIH). Tables 10.1 and 10.2 list the materials, reagents, and solutions required.

1. Euthanize rat with CO_2.
2. Using electric clippers, shave the mid–lower abdomen.
3. Using Nair, a depilatory cream, completely remove the remaining hair per manufacturer's instructions.
4. Place the rat in supine position and stabilize the limbs (Fig. 10.1).
5. Scrub the harvest site with 70% ethanol.

Table 10.1. Preadipocyte Harvest and Isolation Reagents and Materials

Reagents	
Bovine serum albumin (BSA)	Sigma, St. Louis, MO
Ca^{2+}-, Mg^{2+}-free phosphate buffered saline (PBS)	
Collagenase, type IA	Sigma, St. Louis, MO
Dulbecco's Modified Eagle's Medium (DMEM)	
Fetal bovine serum (FBS)	Sigma, St. Louis, MO
Penicillin–streptomycin–glutamine (P/S/G), 100×	Gibco, Gaithersburg, MD
Materials	
0.22-μm Cellulose acetate syringe filter	Costar, Corning, NJ
250-μm Nylon mesh	Sigma, St. Louis, MO
40-μm Cell strainer	Falcon/Becton Dickinson, Franklin Lakes, NJ
50-ml Conical tube	Falcon/Becton Dickinson, Franklin Lakes, NJ
70% Ethanol wetted cotton balls	
Autoclaved, siliconized beaker	
Cell strainer	Sigma, St. Louis, MO
Electric clipper	
Iris scissors	ASSI, Westbury, NY
Mayo dissecting scissors	ASSI, Westbury, NY
Nair Hair Remover	
Tissue forceps (two required)	ASSI, Westbury, NY

Table 10.2. Preadipocyte Harvest and Isolation Solution Preparation

Complete DMEM (cDMEM) (per 500 ml)	350 ml DMEM 5 ml P/S/G 50 ml FBS
Digestion medium (per 2 fat pads)	4 ml 4°C PBS 3 mg/ml Collagenase 3 mg/ml BSA Sterilize through a 0.22-μm syringe filter
PBS with P/S/G	45 ml 4°C PBS 5 ml P/S/G

6. Using dissecting scissors and tissue forceps, aseptically cut through the skin, muscle, and peritoneum along a midline Y-shaped incision, starting from the xiphoid cartilage of the sternum and down and along the inguinal regions of the lower body (Fig. 10.2).
7. Expose the abdominal cavity (Fig. 10.3).
8. Grasp and pull the epididymal adipose tissue (fat pad) with second pair of tissue forceps (Fig. 10.4).
9. Pull the epididymal fat pad until the testis is removed from scrotal sac (Fig. 10.5).
10. Using iris scissors, dissect the fat pad, taking care not to include the internal spermatic artery/vein and caput epididymis (Fig. 10.6).
11. Place harvested fat pad (Fig. 10.7) in a 50-ml conical tube filled with 4°C phosphate-buffered saline (PBS) supplemented with penicillin-streptomycin-glutamino (P/S/G) (Fig. 10.8).

Fig. 10.1. Rat is in supine position, ventral view. Hair of the lower abdomen has been removed via shaving and depilatory cream. Posterior and anterior labels are included to denote rat's head-to-tail orientation.

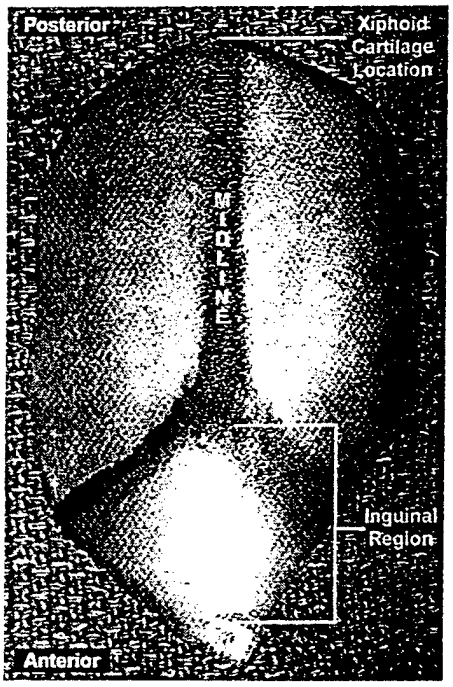


Fig. 10.2. Marking depicting incision to be made through skin, muscle, and peritoneum.

12. Repeat steps 1–11 for the other epididymal fat pad.
13. Place the fat pads in a tissue culture dish in a biosafety cabinet and aseptically remove the large blood vessels (epididymal branch from internal spermatic artery/vein) and any hard tissue (portion of caput epididymis) with scissors and forceps. This minimizes fibroblast contamination of *ex vivo* cultures.
14. Finely mince the tissue with iris scissors.
15. Place the minced tissue into a 50-ml conical tube with digestion medium and then on orbital shaker at a speed of 250 oscillations/min for 20 min at 37°C.
16. Filter the resulting slurry through a 250-μm nylon mesh into a siliconized beaker.
17. Filter the filtrate again through a 40-μm cell strainer into a 50-ml conical tube.
18. Centrifuge the final filtrate at 200*g* for 10 min at 4°C.
19. Aspirate the supernatant and resuspend the pellet (preadipocytes) in warm complete Dulbecco's Modified Eagle's Medium (cDMEM).

Fig. 10.3. Abdominal viscera in situ, *ventral view.*

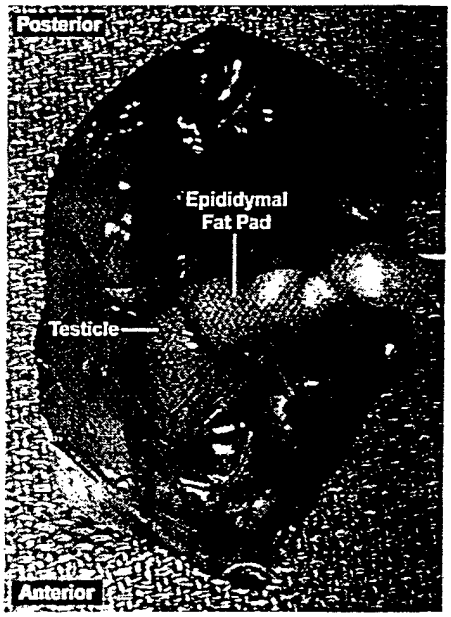


Fig. 10.4. Use of tissue forceps to remove epididymal fat pad.

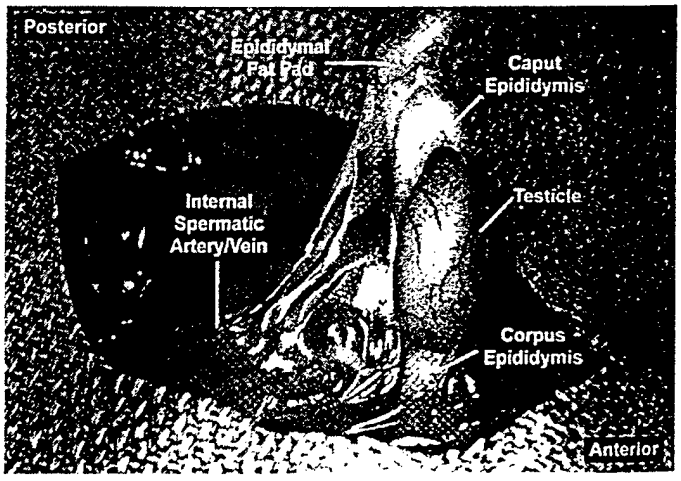


Fig. 10.5. View of epididymal fat pad and surrounding anatomy.

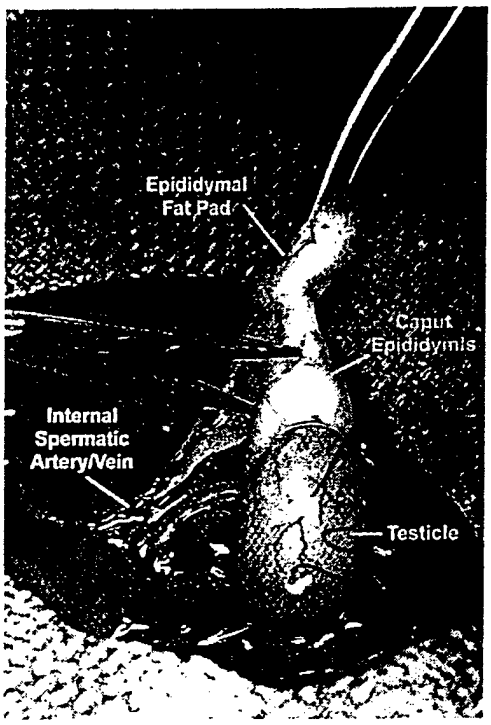


Fig. 10.6. Dissection of epididymal fat pad using iris scissors.

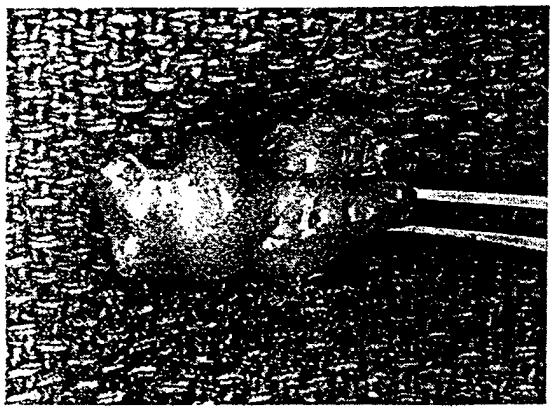


Fig. 10.7. Harvested epididymal fat pad.

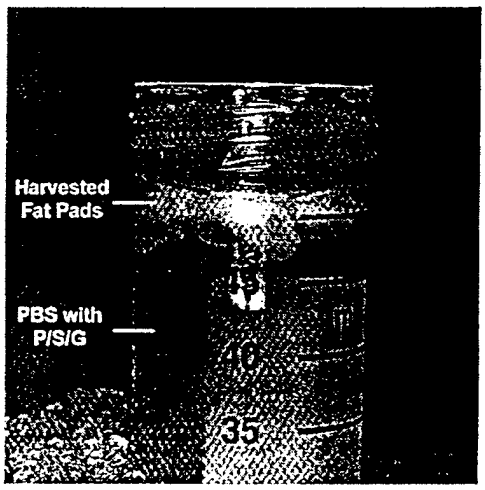


Fig. 10.8. Epididymal fat pads placed in PBS with antibiotics. The 50-ml conical tube is placed in an ice bath.

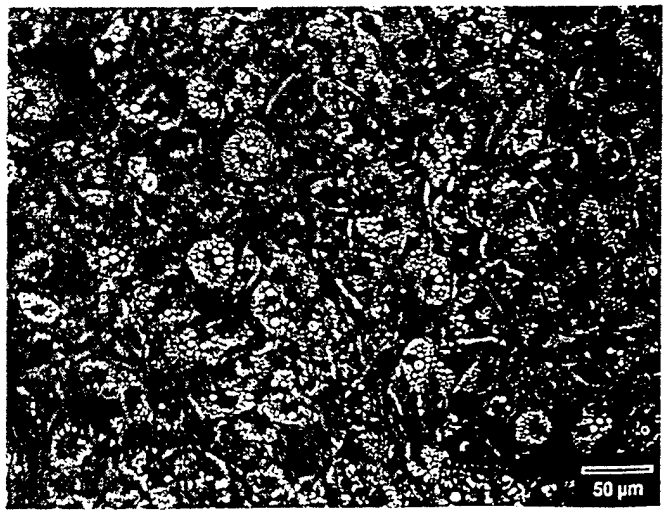
20. Seed the cells into a T75 tissue culture flask. The cell yield is approximately 10^6/ml.
21. Rinse and feed the cells with warm cDMEM after 24 h.
22. Feed the cells every 3 days.

CULTURE OF PREADIPOCYTES

Cells are passaged when the cell density is 90%. At this time, the cell number is approximately 10^7/ml. The preadipocytes are passed prior to confluency, since contact inhibition initiates adipocyte differentiation and ceases preadipocyte proliferation. Cells may be frozen, cold-stored, and thawed in accordance with routine cell culture procedures.

Preadipocytes initially possess a fibroblast-like morphology (Fig. 10.9A). Upon reaching confluency, preadipocytes begin to accumulate lipid pools within their cytoplasm (Fig. 10.9B). Lipid droplets continue to grow in volume and finally coalesce to form a unilocular lipid pool within the cell (Fig. 10.9C–F). At this point, a preadipocyte's cytoplasm is 80–90% lipid. Eventually, the lipid pools become buoyant enough to float mature adipocytes to the surface of the culture flask. The amount of lipid loading can be controlled in a dose-dependent manner by varying the amount of fetal bovine serum (FBS) in cDMEM. Ideally, preadipocytes are seeded into polymers prior to differentiation.

(A)



(B)

Fig. 10.9. Growth and differentiation of rat preadipocytes at five points postseeding: (A) 1 day, (B) 4 days, (C) 7 days, (D) 16 days, and (E) 22 days. Images (A)–(C) are phase contrast; images (D) and (E) are bright field. Note accumulation and coalescence of lipid pools as culture time progresses. (F) Image of unilocular lipid pool within a single preadipocyte. Bars denote 50 µm.

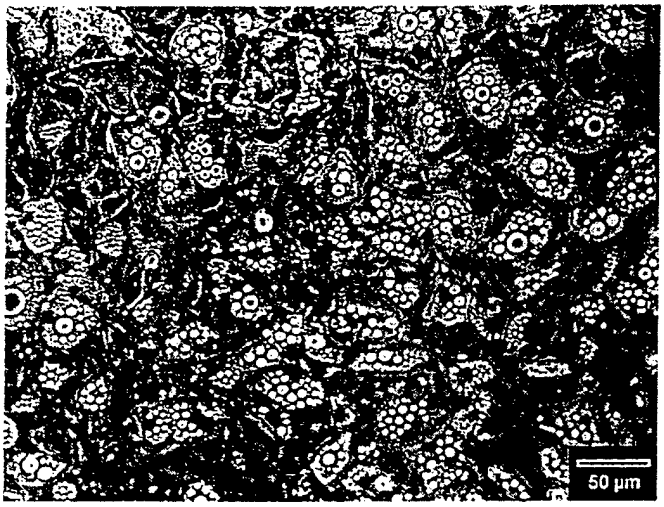
POLYMER SEEDING

The following procedure for seeding poly(lactic-*co*-glycolic acid) (PLGA) polymer foams with preadipocytes [5] has proven successful with other biodegradable polymer foams and nonwoven fibers. Prior to seeding, foams are prewetted and sterilized with absolute ethanol for 30 min followed by two sterile saline washes at 20 min/wash and a DMEM wash for 20 min. A 20-μl suspension of preadipocytes (10^5 cells/mL) is injected onto each foam under sterile conditions. Prewetting permits the cell suspension to readily flow throughout the foam. Following 3 h for cell attachment, 24-well culture plates containing one foam/well are filled with 1.5 ml of medium per well. Foams are ready for implantation.

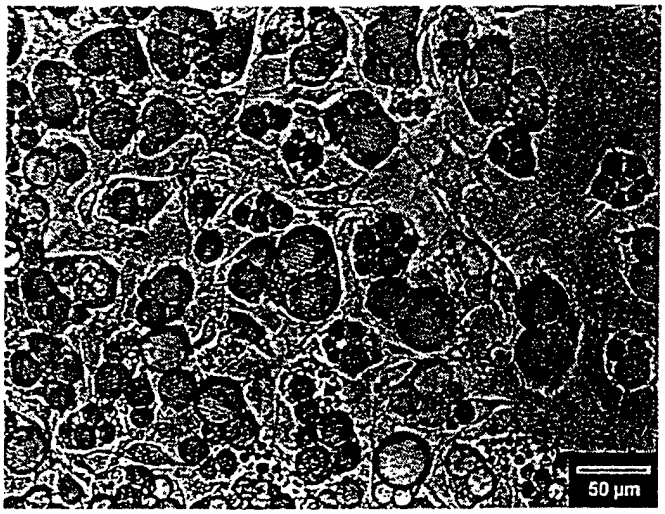
HISTOLOGY

OIL RED O

Adipocyte differentiation *in vitro* is routinely monitored by using oil red O staining for intracellular lipid pools [32] or phase contrast microscopy (lipid appears as phase bright, see Fig. 10.9A–C). The oil red O causes lipid pools to appear red under bright-

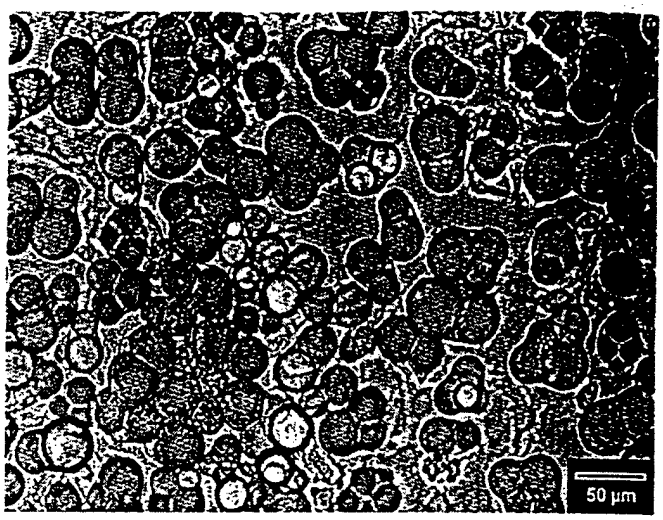


(C)



(D)

Fig. 10.9. (Continued).



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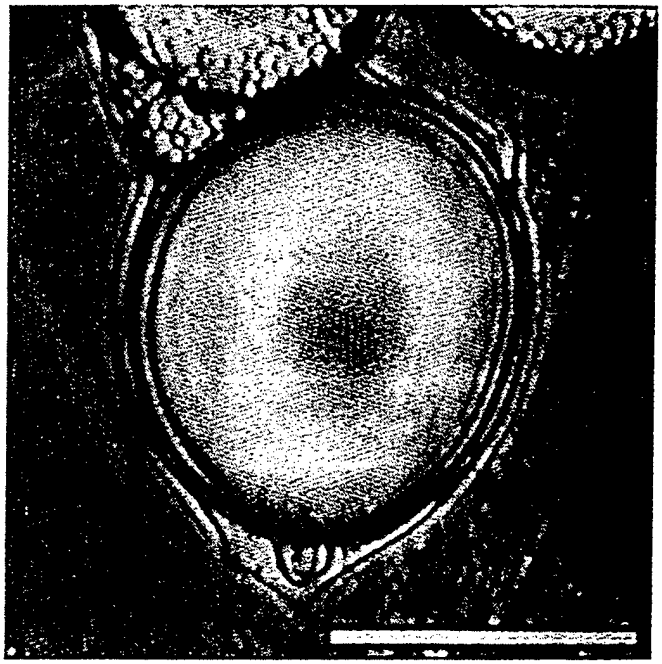


(F)

Fig. 10.9. (Continued).

field microscopy (labeled in Fig. 10.10). Tables 10.3 and 10.4 list the reagents and solutions required. Instructions are as follows.

1. Fix culture with 10% neutral buffer formalin.
2. Rinse with tap water.
3. Stain in oil red O for 10 min.
4. Wash with tap water.
5. Stain for 1 min in acidic Harris hematoxylin.
6. Wash with tap water.
7. Blue in ammonia water.
8. Wash with tap water.
9. Mount with an aqueous mounting medium or acquire pictures immediately. Results: fat, intense red; nuclei, blue.

Fig. 10.10. Preadipocytes in vitro stained with oil red O.

Table 10.3. Oil Red O Reagents

Acetic acid	Fisher Scientific, Pittsburgh, PA
Ammonia hydroxide	Fisher Scientific, Pittsburgh, PA
Aqueous mounting media	Fisher Scientific, Pittsburgh, PA
Harris hematoxylin	Allegiance, McGaw Park, IL
2-Propanol, 98%	Fisher Scientific, Pittsburgh, PA
Neutral buffered formalin, 10%	Fisher Scientific, Pittsburgh, PA
Oil red O	Fisher Scientific, Pittsburgh, PA
Tap water	

Table 10.4. Oil Red O Solution Preparation

Acidic Harris hematoxylin	48 ml Harris hematoxylin 2 ml Acetic acid
Ammonia water	3 ml Ammonia hydroxide 1000 ml water
Oil red O stock solution	2.5 g oil red O 500 ml 2-Propanol Mix well
Oil red O working solution	24 ml oil red O stock solution 16 ml Distilled water Mix well and let stand for 10 min. Filter. The filtrate can be used for several hours

Osmium Petroxide

An osmium tetroxide (OsO_4) paraffin procedure is used to demonstrate fat within harvested *in vivo* polymer foams [33]. Routine staining outlines only "ghost" cells, since histological processing with organic solvents and alcohols extracts lipid from cells. The OsO_4 chemically combines with fat, blackening it in the process. Fat that combines OsO_4 is insoluble in alcohol and xylene, and the tissue can be processed for paraffin embedding and counterstained. Small fat droplets and individual cells are well demonstrated via this method, whereas gross amounts of fat are not fixed by this diffusion-dependent stain (Fig. 10.11).

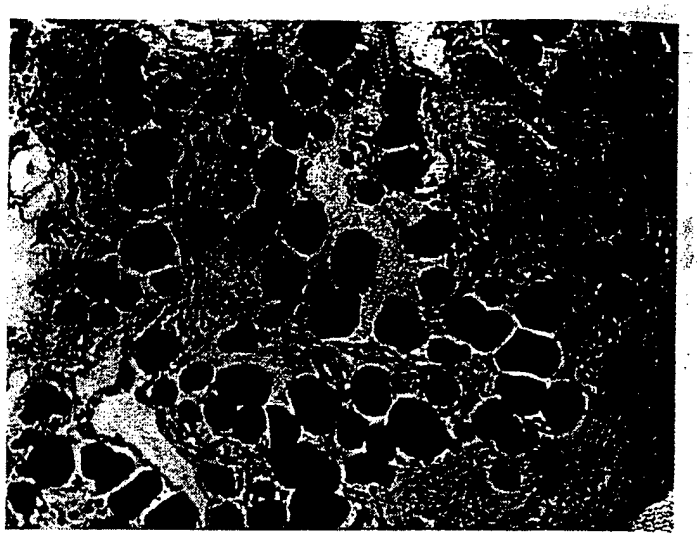


Fig. 10.11. OsO_4-stained preadipocytes.

Table 10.5. OsO_4 Materials and Reagents

Coplin jars	Fisher Scientific, Pittsburgh, PA
Coverslips	Fisher Scientific, Pittsburgh, PA
Distilled water	
Formalin, 10%	Fisher Scientific, Pittsburgh, PA
Tap water	
Osmium tetroxide (OsO_4), 1% (wt/vol), aq	Sigma, St. Louis, MO
Pencil	
Periodic acid, 0.5% (wt/vol), aq	Sigma, St. Louis, MO
Rotating shaker	Fisher Scientific, Pittsburgh, PA
Slides	Fisher Scientific, Pittsburgh, PA
Standard paraffin embedding cassettes	Fisher Scientific, Pittsburgh, PA

After staining with OsO_4, foams are processed for paraffin embedding using standard procedures, except that HistoSolve (Shandon Lipshaw), a xylene substitute, is used instead of xylene. Xylene dissolves many biodegradable polymers. Infiltrated foams are cut and oriented in embedding cassettes. Sections 6 μm thick are cut with a microtome (Leica, Wetzlar, Germany), placed on slides, stained with hematoxylin–eosin (H&E), and coverslipped. Sections are analyzed by means of bright-field microscopy (Fig. 10.11). Table 10.5 lists the materials and reagents required. Instructions are as follows.

1. Harvest polymer foams at the appropriate time from rats.
2. Place harvested foams directly in a vial containing 10 ml of 10% formalin and fix overnight at room temperature.
3. After 24 h, remove specimen from formalin and trim. If cross sections are desired, a small piece of the foam to be embedded in cross section is removed at this time.
4. Transfer specimens to individual embedding cassettes that have been appropriately labeled with a pencil.
5. Place all cassettes in a beaker under freely running tap water and wash for 1 h.
6. Wash for 1 h in freely running distilled water.
7. Transfer cassettes to a Coplin staining jar (4 cassettes per jar) containing 5 ml of 1% aqueous OsO_4 solution.
8. Cap the jars and place on rotating shaker at 60 rpm under a fume hood at room temperature for 1 h.

9. Dispose of the OsO_4 in a hazardous waste container and rinse samples for 30 min in freely running distilled water.
10. Differentiate tissue by placing it in 0.5% aqueous periodic acid for 30 min on rotating shaker at 60 rpm.
11. Wash samples in running tap water for 30 min.
12. At this point samples are ready to be processed for paraffin embedding. Proceed with normal processing procedure using a xylene substitute instead of xylene and counterstain with H&E. Results: fat, black.

REFERENCES

[1] Patrick, C. W., Jr., Chauvin, P. B., and Robb, G. L. (1998). Tissue engineered adipose. *In* "Frontiers in Tissue Engineering," pp. 369–382. Elsevier Science, Oxford.

[2] Katz, A. J., Llull, R., Hedrick, M. H., and Futrell, J. W. (1999). Emerging approaches to the tissue engineering of fat. *Clin. Plast. Surg.* 26, 587–603.

[3] Kral, J. G., and Crandall, D. L. (1999). Development of a human adipocyte synthetic polymer scaffold. *Plast. Reconstr. Surg.* 104, 1732–1738.

[4] Kawaguchi, N., Toriyama, K., Nicodemou-Lena, E., Inou, K., Torii, S., and Kitagawa, Y. (1998). De novo adipogenesis in mice at the site of injection of basement membrane and basic fibroblast growth factor. *Cell Biolog.* 95, 1062–1066.

[5] Patrick, C. W., Jr., Chauvin, P. B., and Reece, G. P. (1999). Preadipocyte seeded PLGA scaffolds for adipose tissue engineering. *Tissue Eng.* 5, 139–151.

[6] MacDougald, O. A., and Lane, M. D. (1995). Transcriptional regulation of gene expression during adipocyte differentiation. *Ann. Rev. Biochem.* 64, 345–373.

[7] Smas, C. M., and SookSul, H. S. (1995). Control of adipocyte differentiation. *Biochem. J.* 309, 697–710.

[8] Butterwith, S. C. (1994). Molecular events in adipocyte development. *Pharm. Ther.* 61, 399–411.

[9] Cornelius, P., MacDougald, O. A., and Lane, M. D. (1994). Regulation of adipocyte development. *Annu. Rev. Nutr.* 14, 99–129.

[10] Brun, R., Kim, J., Hu, E., Altiok, S., and Spiegelman, B. (1996). Adipocyte differentiation: A transcriptional regulatory cascade. *Cell Differ.* 8, 826–832.

[11] Sul, H. S., Smas, C. M., Wang, D., and Chen, L. (1998). Regulation of fat synthesis and adipose differentiation. *In* "Progress in Nucleic Acid Research and Molecular Biology," pp. 317–345. Academic Press, Berkely, CA.

[12] Fukai, F., Iso, T., Sekiguchi, K., Miyatake, N., Tsugita, A., and Katayama, T. (1993). An amino-terminal fibronectin fragment stimulates the differentiation of ST-13 preadipocytes. *Biochemistry* 32, 5746–5751.

[13] Spiegelman, B. M., and Ginty, C. A. (1983). Fibronectin modulation of cell shape and lipogenic gene expression in 3T3-adipocytes. *Cell (Cambridge, Mass.)* 35, 657–666.

[14] Pairault, J., and Green, H. (1979). A study of the adipose conversion of suspended 3T3 cells by using glycerophosphate dehydrogenase as differentiation marker. *Proc. Natl. Acad. Sci. U.S.A.* 76, 5138–5142.

[15] Casimir, D. A., Miller, C. W., and Ntambi, J. M. (1996). Preadipocyte differentiation blocked by prostaglandin stimulation of prostanoid FP^2 receptor in murine 3T3-L1 cells. *Differentiation (Berlin)* 60, 203–210.

[16] Green, H., and Kehinde, O. (1979). Formation of normally differentiated subcutaneous fat pads in an established preadipocyte cell line. *J. Cell Physiol.* 101, 169–171.

[17] Green, H., and Kehinde, O. (1975). An established preadipose cell line and its differentiation in culture II. Factors affecting the adipose conversion. *Cell (Cambridge, Mass.)* 5, 19–27.

[18] Green, H., and Meuth, M. (1974). An established pre-adipose cell line and its differentiation in culture. *Cell (Cambridge, Mass.)* 3, 127–133.

[19] Marko, O., Cascieri, M. A., Ayad, N., Strader, C. D., and Candelore, M. R. (1995). Isolation of a preadipocyte cell line from rat bone marrow and differentiation to adipocytes. *Endocrinology (Baltimore)* 136, 4582–4588.

[20] Kirkland, J. L., Hollenberg, C. H., Kindler, S., and Gillon, W. S. (1994). Effects of age and anatomic site on preadipocyte number in rat fat depots. *J. Gerontol.* 49, B31–B35.

[21] Kirkland, J. L., Hollenberg, C. H., and Gillon, W. S. (1996). Effects of fat depot site on differentiation-dependent gene expression in rat preadipocytes. *Int. J. Obes. Relat. Metab. Disord.* 20(Suppl. 3), S102–S107.

[22] Djian, P., Roncari, D. A. K., and Hollenberg, C. H. (1983). Influence of anatomic site and age on the replication and differentiation of rat adipocyte precursors in culture. *J. Clin. Invest.* 72, 1200–1208.

[23] Stanton, L. A., von Venter, M., Litthauer, D., and Oelofsen, W. (1997). Effect of lipoproteins on the differentiation of 3T3-L1 and human preadipocytes in cell culture. *Comp. Biochem. Physiol.* 116B, 65–73.

[24] Teichert-Kuliszewska, K., Hamilton, B. S., Roncari, D. A. K., Kirkland, J. L., Gillon, W. S., Deitel, M., and Hollenberg, C. H. (1996). Increasing vimentin expression associated with differentiation of human and rat adipocytes. *Int. J. Obes.* 20(Suppl. 3), S108–S113.

[25] Barbe, P., Galitzky, J., Gilsezinski, I. D., Riviere, D., Thalamas, C., Senard, J. M., Crampes, F., Lafontan, M., and Berlan, M. (1998). Simulated microgravity increases B-adrenergic lipolysis in human adipose tissue. *Clin. Endocrinol. Metab.* 83, 619–625.

[26] O'Brien, S. N., Mantzke, K. A., Kilgore, M. W., and Price, T. M. (1996). Relationship between adipose stromal-vascular cells and adipocytes in human adipose tissue. *Anal. Quant. Cytol. Histol.* 18, 137–143.

[27] Poznanski, W. J., Waheed, I., and Van, R. (1973). Human fat cell precursors: Morphologic and metabolic differentiation in culture. *Lab. Invest.* 29, 570–576.

[28] Novakofski, J. E. (1987). Primary cell culture of adipose tissue. *In* "Biology of the Adipocyte: Research Approaches," pp. 160–197. Van Nostrand-Reinhold, New York.
[29] Hauner, H., Entenmann, G., Wabitsch, M., Gaillard, D., Aihaud, G., Negrel, R., and Pfeiffer, E. F. (1989). Promoting effect of gluctosteroids on the differentiation of human adipocyte precursor cells cultured in a chemically defined medium. *J. Clin. Invest.* 84, 1663–1670.
[30] Entenmann, G., and Hauner, H. (1996). Relationship between replication and differentiation in cultured human adipocyte precursor cells. *Am. J. Physiol.* 270, C1011–C1016.
[31] Strutt, B., Khalil, W., and Killinger, D. (1996). Growth and differentiation of human adipose stromal cells in culture. *In* "Methods in Molecular Medicine: Human Cell Culture Protocols," pp. 41–51. Humana Press, Totowa, NJ.
[32] Carson, F. L. (1997). *In* "Histotechnology," pp. 152–153. ASCP Press, Chicago.
[33] Carson, F. L. (1997). *In* "Histotechnology," pp. 153–154. ASCP Press, Chicago.

CHAPTER 78

BREAST RECONSTRUCTION

Geoffrey L. Robb, Michael J. Miller, and Charles W. Patrick Jr.

INTRODUCTION

Breast cancer continues to be the most common cancer among women, other than cancers of the skin, with an incidence of nearly one of every three cancers occurring in American women. Approximately one in nine women in this country will develop breast cancer by 85 years of age. The United States has the highest incidence of breast cancer in the world, with 110.6 cases per 100,000 women. In the year 2000, roughly 182,800 new cases of invasive breast cancer were diagnosed.

From a surgical therapeutic perspective, 69,683 women underwent breast reconstruction in 1998, a 135% increase since 1992. This represents 3.1% of all plastic surgery procedures for that year. The majority of patients, 49%, were in the 35- to 50-year-old age group, while 36% were in the 51- to 64-year-old age group. Thirty-nine percent of the reconstructions were performed at the same time as the mastectomy, which represents the immediate use of both implants in 46% of patients as well as autogenous tissues in 37% of patients for breast reconstruction [1].

These statistics underscore the growing importance of cancer rehabilitation in the form of breast reconstruction for women affected by breast cancer in all age groups. As opposed to being an issue of mere vanity, restoration of the breast form and contour is valued for the necessary maintenance of self-esteem and body image. Even limited excisions of the breast for the eradication of cancer can produce permanent breast deformities and breast asymmetry. The relevant importance of this "woman's issue" was recently supported by the passage by both houses of Congress of the Omnibus Budget Bill. In this bill, insurance companies are required not only to underwrite reconstructive breast surgery following cancer treatment, but also to cover the additional procedures necessary to maintain symmetry with the opposite normal breast. There is a clear ethical and personal mandate to support the reconstruction of breast deformity, whether congenital, secondary to trauma, or, in particular, following cancer treatment.

TYPES OF BREAST RECONSTRUCTION

Plastic surgery for the breast may be broadly classified as either primarily reconstructive or aesthetic depending on the nature of the deformity. This distinction is primarily one of degree. Cosmetic procedures address deformities that are anatomically within normal limits but nevertheless present an appearance that is unsatisfactory to the patient. Aesthetic breast enlargement surgery, or augmentation mammaplasty, is one of the most common aesthetic procedures. Several hundred cubic centimeters of additional soft tissue or tissue equivalent may be required. Usually this is supplied by breast implants consisting of an envelope made of silicone elastomer filled with either saline solution or silicone gel. The ability to engineer additional fat would potentially eliminate the need for artificial breast implants. Reconstructive operations correct more extreme problems; however, they must still follow proper aesthetic principals. After all, the most sophisticated breast reconstruction that does not look like a normal breast will not be well accepted. Both reconstructive and aesthetic oper-

ations may require tissue replacement and therefore may be influenced by developments in tissue engineering.

Breast reconstruction is one of the most common reconstructive procedures. The usual indication is to restore the breast following complete removal (i.e., total mastectomy) performed for cancer treatment. It has been shown that women with breast cancer must deal with two separate emotional issues, the reality of a life-threatening disease and the possibility of losing a breast. Loss of the breast is not life-threatening, but many women find the deformity emotionally and psychologically disturbing [2]. A mastectomy causes a significant functional and cosmetic deformity, replacing the soft, projecting breast with a long, flat scar. The breast is a significant part of female body image and sense of femininity. The patient is reminded of her cancer experience every time she looks in a mirror. It can be difficult to find clothing. For some women, an external prosthesis may be satisfactory, but many find such devices cumbersome and unacceptable. Breast reconstruction is intended to overcome these problems and enhance the quality of life for women following mastectomy for breast cancer. The structure of the female breast consists of a container made of skin filled with soft glandular and fatty tissue.

Postmastectomy breast reconstruction involves replacing missing skin and soft-tissue volume to recreate the appearance of the breast. Current methods rely on breast implants [3], soft-tissue flaps [4], or a combination of these [5]. Each technique offers certain advantages depending on the patient. Reconstruction based primarily on artificial devices uses a process known as tissue expansion to create additional skin. This involves placing an inflatable silicone device called a tissue expander beneath the tissues. The expander is gradually inflated with physiological saline solution injected over several weeks. When the expansion is complete, the device is removed and the tissue is ready to use. This process has been shown to increase the amount of tissue and improve the blood supply. It requires up to 6 months to complete. The tissue expander is then replaced with a permanent implant to provide the necessary volume for the completed reconstruction.

Autologous tissue reconstruction is most often performed by means of skin and fat obtained from the lower anterior abdominal wall as a flap, called a transverse rectus abdominis musculocutaneous (TRAM) flap (Fig. 78.1). The tissue may be transferred by either keeping the rectus abdominis muscle attached superiorly or by performing a microvascular transfer [6]. The skin and fat may then be shaped to simulate the appearance of the breast. The tissue is a similar in consistency to breast tissue and provides a reconstructed breast that looks and feels the most natural. The transverse scar at the donor site, located midway between the umbilicus and the pubic area, is easily hidden by clothing. When there is inadequate tissue on the lower abdomen, the procedure can be combined with placement of a breast implant. In such cases, tissue may be harvested from either the back or the abdomen. If tissue is used, however, the results tend to be more natural and long-lasting. The disadvantages of these operations are that they are more time-consuming, have greater risks, and cause more scarring than other techniques.

There are advantages minimizing dependence on permanent breast implants for breast reconstruction. Implants can erode through the skin, become infected, and form deforming scars. They are more difficult to control during the shaping and contouring and can create unnatural surface contours over time. Breast implants are not a good option in patients who have been treated with radiation because of a tendency for firm scars to form around the implant. Reconstruction based entirely on tissue avoids these problems, but requires surgery other sites on the patient, resulting in alteration of normal areas. It is the opportunity to achieve a natural tissue reconstruction without donor site problems that provides the incentive to develop fat-tissue approaches to breast reconstruction. A tissue-engineered soft-tissue alternative would have wide application in postmastectomy breast reconstruction.

SURGICAL PRINCIPLES AND TISSUE ENGINEERING

To envision how tissue engineering methods might be applied to breast reconstruction, it is helpful to consider some surgical principles. The use of tissue in reconstructive surgery involves a two-step process of *transfer* from an uninjured location (donor site) and *modification* to replace or simulate the breast tissue that which has been lost (Fig. 78.1).

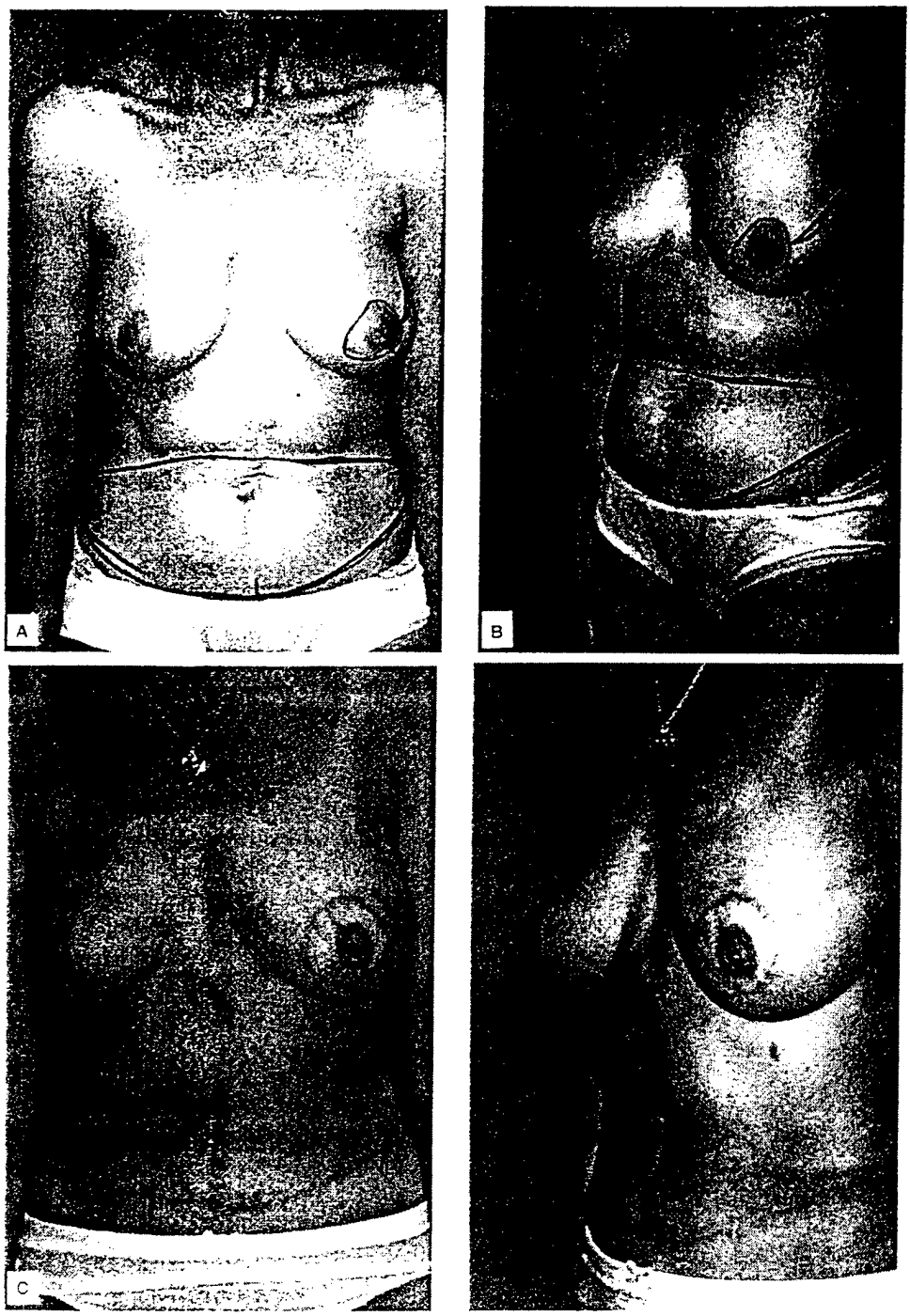


Fig. 78.1. (a) Preoperative skin-sparing mastectomy utilizing larger TRAM flap designowing to midline lower abdominal scar; (b) preoperative free TRAM reconstruction using skin-sparing mastectomy; (c) postoperative free TRAM reconstruction, nipple–areolar reconstruction completed; and (d) postoperative free TRAM reconstruction.

Tissue Transfer

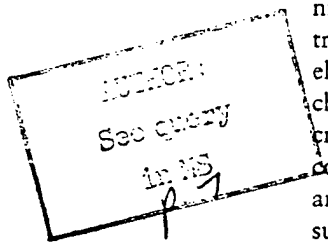
Tissue transfer methods may be classified as either tissue grafts or flaps. A graft is any tissue transferred without its blood supply. Graft healing depends upon nutritive support passively available in the tissues surrounding the graft. Small amounts of skin, dermis, and fat may be transferred in this way. In breast reconstruction, these tissues are autologous, or obtained from an uninjured location on the same patient. The volume of tissue required for breast reconstruction is sufficiently large that it cannot survive transfer as a graft.

When a large amount of tissue is required, it must be transferred with a blood supply that originates from outside the zone of injury. This is the definition of a surgical "flap," the traditional term for a unit of tissue moved to another location with preservation of its blood supply. Tissue transferred as flaps may be moved into a compromised area because they will not depend on the ability of the surrounding tissues to supply nutritive support. A variety of surgical flaps have been described that provide skin and fat suitable for breast reconstruction. The most common, however, is located on the lower abdomen. The blood supply to this area passes through the rectus abdominis muscle. It is therefore possible to move the skin and fat of the lower abdomen to the chest for breast reconstruction, using the rectus muscle as a conduit for the blood supply. The most advanced transfer technique is a microvascular transfer. This method involves isolating the tissue unit on its primary vascular supply and temporarily dividing the blood vessels, cutting it "free" from the patient. Tissues transferred in this way are often called "free flaps." The vessels supplying the flap, usually 1–3 mm in diameter, are sewn with extremely fine suture materials to other vessels near the defect; the surgoon is aided by an operating microscope. Usually, two microvascular anastomoses are required, one for the artery and one for the vein. Skin and fat transferred in this way heal in the normal way with little contracture or loss of substance. Most of the history of plastic surgery consists of advances in techniques to transfer tissue.

Tissue Modification

Tissue modification is the second step of the reconstruction. After transfer, tissues must be reshaped to simulate missing structures. In contrast to tissue transfer techniques, tissue modification methods have changed little through the centuries. Surgeons still learn to manually alter tissues at the time of surgery, an often difficult and time-consuming process. The results are never exact. Original breast is approximated to a degree that varies depending on the nature of the tissues and the personal skill of the surgeon. The primary factor limiting these methods is the need to preserve adequate blood supply to all portions of the tissue. Efforts to overcome these limitations have focused improving the blood supply and "prefabricating" structures prior to transfer [7–14].

More elaborate methods of tissue modification leading up to tissue engineering have been described experimentally and used in selected patients. As early as 1963, attempts were made to revascularize tissues by direct transfer of blood vessels [15–17]. These techniques are based upon rearranging mature tissue elements into useful configurations prior to transfer. Even more advanced than simple rearrangement is direct modification of the tissue elements by using implants and induction factors to mold and transform. Hollow molding chambers made from inert titanium or silicone have been used in laboratory animals to create tissue flaps of different shapes [18,19]. Khouri *et al.* added a potent growth factor to control the differentiation of soft tissue inside molding chambers placed inside laboratory animals [20]. Despite these laboratory studies that demonstrate the feasibility of fabricating surgical flaps into different shapes, clinically useful techniques have yet to emerge. Only small amounts of tissue have been produced, and the proper shape has not been retained after removal of the mold.



The goal of tissue engineering is to improve our ability to modify tissues by shifting from working with whole tissues to more fundamental levels. From a surgeon's viewpoint, tissue engineering is modification of existing tissues at the cellular or molecular level to fabricate new tissues for reconstructive surgery.

TISSUE ENGINEERED BREAST EQUIVALENT

Preface

The application of tissue engineering to breast fabrication is a relatively new effort. The following sections give an overview of the state of the art and the preliminary attempts in this new venue of tissue engineering. The guiding concept is to develop a vascular construct to restore the breast mound and provide optimum cosmesis such that the limitations with tissue transfer and breast implants are abrogated. Strategies for developing tissue constructs within the breast envelope and *ex vivo* followed by subsequent implantation into the breast envelope are being investigated [21–24]. Restoring functional aspects of the breast, such

as lactation and tactile stimulation, are beyond the scope of current strategies. However, investigators have utilized tissue engineering strategies for nipple reconstruction [25].

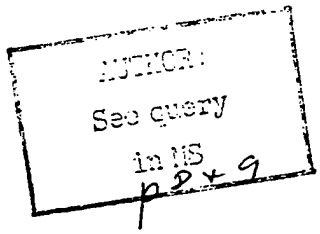
To be sure, the development of a breast equivalent is particularly challenging. Unlike most other tissues and organs, breast tissue is highly variable among patients with respect to volume, composition, shape, soft-tissue biomechanics, ethnicity, age, and hormonal environment (i.e., pre-/postmenopause, pregnancy). Moreover, the final aesthetic outcome of a breast strongly affects the emotional well-being of a patient. In addition, breast aesthetics truly follow the platitude "beauty is in the eye of the beholder," and patient expectations often overrule a surgeon's concept of the individual's optimum breast.

Tissue engineering modalities can be segregated into four fundamental components, namely, cells, scaffold, microenvironment, and elucidation of patient-specific design parameters. Each is discussed here under the aegis of breast tissue engineering.

Cells for a Breast Equivalent

Adipose

A breast largely consists of adipose tissue setting in the skin envelope against the pectoral muscles of the chest wall. Naturally, the development of a tissue equivalent for breast restoration has focused on human adipose tissue. Adipose tissue is ubiquitous, the largest tissue in the body, uniquely expendable, and most patients possess excess that can be harvested without creating contour deformities. Autologous fat transplantation gives poor results, with 40–60% reduction in graft volume [22,26,27]. The reduction in adipose volume is postulated to be related to insufficient revascularization. The advent of liposuction led investigators to attempt using single-cell suspensions of mature adipocytes. However, since adipocytes possess a cytoplasm composed of 80–90% lipid, they readily tend, upon aspiration, to be traumatized by the mechanical forces of liposuction, resulting in about 90% damaged cells. The remaining 10% tend to form cysts or localized necrosis postinjection. Moreover, mature adipocytes cannot be expanded *ex vivo* because they are terminally differentiated.



Recent progress has been made by using preadipocytes, precursor cells that differentiate into mature adipocytes. Preadipocytes are fibroblast-like cells that uptake lipid during differentiation. They grow easily with standard cell culture technologies, they can be expanded *ex vivo*, and the molecular biology involved in differentiation has largely been elucidated through research in the obesity and diabetes areas [28,29]. However, much of the application-based biology of preadipocytes remains unknown (e.g., cell adhesion, cell motility, response to various microenvironments). Human, rat, and swine preadipocytes have been routinely cultured [30–35]. Preadipocytes are normally isolated from enzyme-digested adipose tissue or liposuction material [22]. Alternatively, adipocyte stem cells may potentially allow one to develop cultures of preadipocytes. Researchers are predominantly focusing on using subcutaneous preadipocytes for tissue engineering strategies. It is known that fat depots at different anatomical locations behave differently [36–39]. Hence, it remains to be seen if subcutaneous preadipocytes can adequately replace mammary adipose.

Microvascular Network

Any potential clinically translatable tissue engineering modality must consider the microvasculature. Adipose tissue is unique in that it has the capacity to continue to grow and its vascular network grows in tandem (i.e., *de novo* angiogenesis) [40]. Adipose tissue is highly vascular. The capillary density of adipose is approximately one-third that of muscle. However, from a metabolism standpoint and correcting for active protoplasm (i.e., since an adipocyte is largely lipid within its cytoplasm) the capillary bed of adipose is far richer (~ two or three times) than that of muscle. Adipose tissue is also known to enhance angiogenesis through the secretion of growth factors extracellular matrices (ECMs) [41–43].

Of the three biological mechanisms available to vascularize a tissue equivalent, only two are available to adults, namely, revascularization and inosculation. Revascularization denotes the growth of capillaries from a host site or tissue into a tissue equivalent. Except for relatively thin constructs, which can survive by diffusion, the slow kinetics (on the order of weeks) of this process typically abrogates it use for large constructs. It has

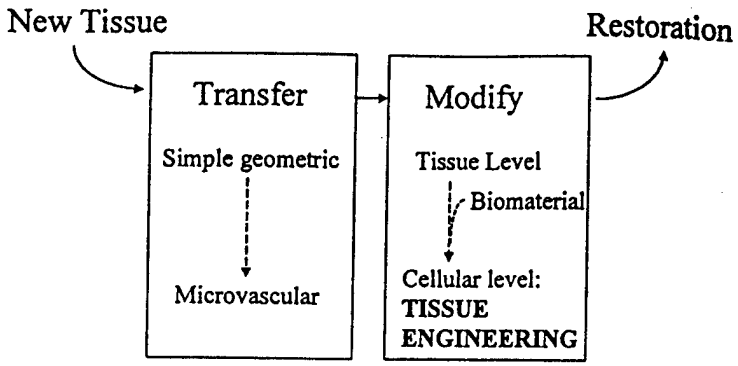


Fig. 78.2. Tissue engineering viewed in the context of the process of reconstructive surgery may be considered in advance in the step of tissue modification.

been proposed to use the highly vascular and adipocyte-rich omentum to encase constructs [44–46]. Inosculation is the process of two capillaries or capillary networks fusing together. The kinetics of inosculation occurs on the order of hours and is the predominant factor that allows plastic surgeons to transfer tissue from a donor site to a recipient site. The capillary networks of the recipient site and the graft fuse together, thus forming a patent vascular network throughout the graft. The use of inosculation in a tissue engineering strategy requires either the seeding of microvascular endothelial cells into or the *ex vivo* or *in situ* development of capillary networks within a tissue equivalent. Both modalities are being investigated but are currently hindered by the lack of understanding of the biological mechanisms that control inosculation and of understanding of capillary formation and cell culture technology of microvascular endothelial cells. Knowledge gained by using vein- or artery-derived endothelial cells cannot be directly translated to capillary endothelial cells.

Scaffolds

A support structure is required for anchorage-dependent cells to migrate and proliferate and to give a tissue equivalent the boundary conditions for final overall tissue shape. Implantable materials utilized have predominantly been porous biodegradable polymer foams [21–23]. For instance, poly(actic-*co*-glycolic acid) (PLGA) scaffolds preseeded with preadipocytes have demonstrated adipose tissue formation [21]. Polymer foams, however, will probably not be the optimum choice for breast scaffolds: they are too rigid for the breast envelope and would be uncomfortable for the patient. In 1999 Kral and Crandall used a non biodegradable scaffold to demonstrate the attachment and proliferation of preadipocytes on Fluorotex monofilament-expanded poly(tetrafluoroethylene) scaffolds coated with various ECMs [47]. Injectable materials, such as hydrogels, inherently possess optimum properties for use in the breast envelope. Both alginate and hyaluronic acid gels have been investigated [23,48,49]. In addition, preadipocytes successfully proliferated and differentiated within fibrin gels.

Finally, adjustable implants have been proposed. Vacanti and colleagues have conceptualized serial injections of a cell-seeded hydrogel within a tissue expander device, with the tissue expander being decreased in size each time an injection is conducted [50]. The optimum scaffold for breast tissue engineering remains elusive. Derivatizing polymers with adhesion molecules can potentially optimize scaffolds. However, this strategy is complicated by the variation of the constitution and distribution of the ECM during adipocyte differentiation [51]. Although short-term studies have demonstrated adipose formation within biodegradable polymers, it remains to be determined whether the formed adipose tissue resorbs over the long term. Investigators are involved in a year-long study to determine the sustainability of tissue engineered adipose [C. W. Patrick, Jr., unpublished data].

Adipose Microenvironment

The microenvironment surrounding a tissue construct affects its differentiation and rate of tissue formation. Adipogenesis can be affected, in part, by growth factors (endogenous and exogenous), pO_2 (normoxia vs hypoxia), pH, adhesion molecule on ECM and support cells, and micromotion. Kawaguchi *et al.* demonstrated *de novo* adipogenesis following in-

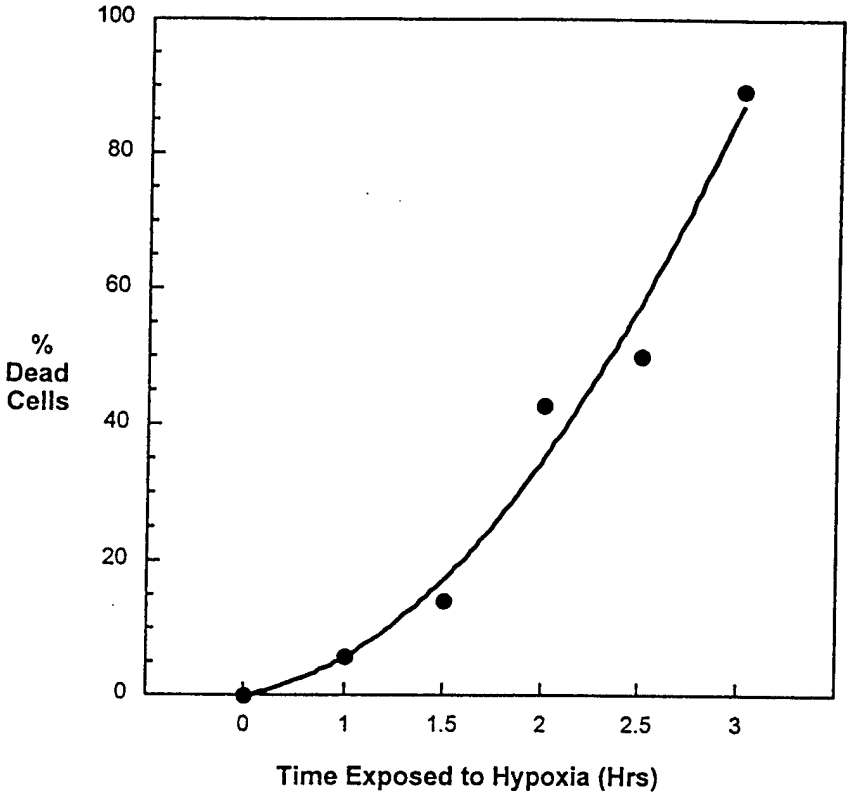


Fig. 78.3. Viability of rat preadipocytes exposed to a hypoxic environment (0% O_2).

jection of Matrigel and basic fibroblast-like growth factor (bFGF) in mice [52]. Yuksel and colleagues have used biodegradable microspheres loaded with insulin, bFGF, and insulin-like growth factor 1 to differentiate preadipocytes to mature adipocytes *in vivo* [53,54]. In addition, both epidermal growth factor and tumor necrosis factor α inhibit adipose differentiation [55,56]. Preadipocytes are extremely sensitive to hypoxic environments (Fig. 78.3). This is not surprising based on the historical results of free fat grafting. It is a major design constraint, however, insofar as it limits the time preadipocytes can be placed in a breast envelope without an adequate microvascular network. In contrast, microvascular endothelial cells have been shown to survive hypoxic conditions for 5–7 days.

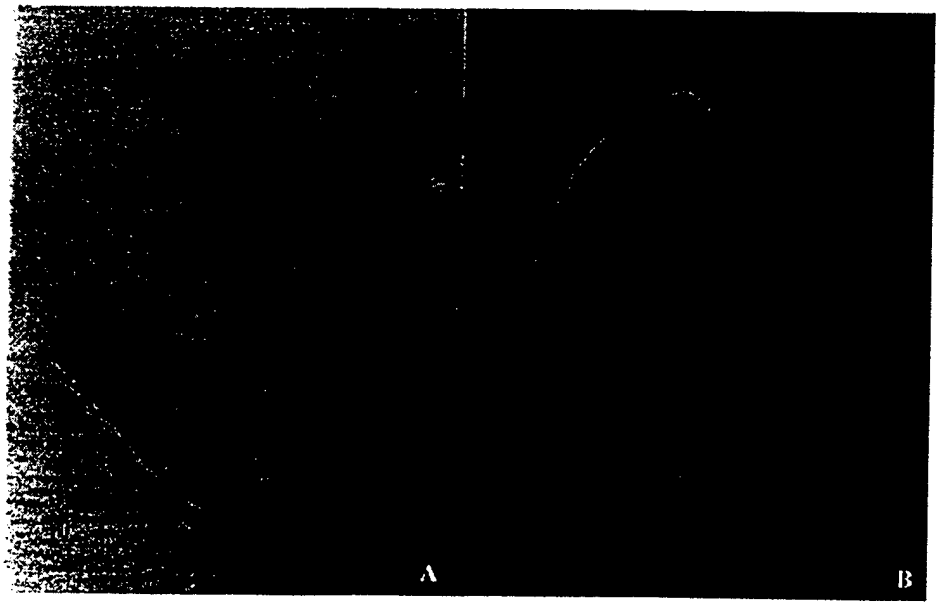


Fig. 78.4. Virtual reality breast simulator. (a) Range data of a patient's breast (three-dimensional surface scan). (b) Fitted virtual breast model, resulting in a volume of 987 ml and surface area of 453 cm^2.

Patient-Specific Design Parameters

To be truly clinically translatable a breast tissue engineering strategy must be patient specific. Unlike strategies for organs that can largely be grown as "one size fits all," breast shape and volume vary widely among the patient population. Breast implants, for instance, range from 100 ml to 2 liters. Hence, methods must exist to predetermine design parameters preoperatively such that the final outcome is known *a priori*. To accomplish this goal, bioengineers, physicians, and computer scientists have combined skill sets to develop a virtual reality breast simulator. A first-generation VR model of the female breast has been developed (Fig. 78.4) The system uses a global parametric deformable model of an ideal breast and allows the surgeon to manipulate the shape of the breast by varying five key shape variables, analogous to the aesthetic and structural elements surgeons inherently vary manually during breast reconstruction. The variables are ptosis (sagging of the breast), top-shape (top's concavity/convexity), turn-top (orientation of top half of the breast with respect to the shoulders), flatten-side (side's concavity/convexity), and turn (deflection of nipple orientation from a perpendicular axis originating at the chest wall). The second generation of the VR model is being developed to be patient specific by importing three-dimensional measurements of the surface of a patient's breast obtained via surface scanning.

References

1. Robb, G. L. (2000). Breast reconstruction. Internet Site: YourDoctor.com.
2. Gilboa, D., Borenstein, A., Floro, S., *et al.* (1990). Emotional and psychological adjustment of women to breast reconstruction and detection of subgroups at risk for psychological morbidity. *Ann. Plast. Surg.* 25, 397–401.
3. Cohen, B. E., Casso, D., and Whetstone, M. (1992). Analysis of risks and aesthetics in a consecutive series of tissue expansion breast reconstructions. *Plast. Reconstr. Surg.* 89, 840–843.
4. Bostwick, J., and Jones, G. (1994). Why I choose autogenous tissue in breast reconstruction. *Clin. Plast. Surg.* 21, 165.
5. Fisher, J., and Hammond, D. (1994). The combination of expanders with autogenous tissue in breast reconstruction. *Clin. Plast. Surg.* 21, 309.
6. Schusterman, M. A., Kroll, S. S., and Weldon, M. E. (1992). Immediate breast reconstruction: Why the free TRAM over the conventional TRAM? *Plast. Reconstr. Surg.* 90, 255–261.
7. Özgenta, H. E., Shenaq, S., and Spira, M. (1995). Prefabrication of a secondary TRAM flap. *Plast. Reconstr. Surg.* 95, 441–449.
8. Itoh, Y. (1992). An experimental study of prefabricated flaps using silicone sheets, with reference to the vascular patternization process. *Ann. Plast. Surg.* 28, 140–146.
9. Mulliken, J. B., and Glowacki, J. (1980). Induced osteogenesis for repair and construction in the craniofacial region. *Plast. Reconstr. Surg.* 65, 553–560.
10. Hirase, Y., Valauri, F. A., and Buncke, H. J. (1988). Prefabricated sensate myocutaneous and osteomyocutaneous free flaps: An experimental model. Preliminary report. *Plast. Reconstr. Surg.* 82, 440–445.
11. Stark, G. B., Hong, C., and Futrell, J. W. (1987). Enhanced neovascularization of rat tubed pedicle flaps with low perfusion of the wound margin. *Plast. Reconstr. Surg.* 80, 814–824.
12. Hussl, H., Russell, R. C., Zook, E. G., and Eriksson, E. (1986). Experimental evaluation of tissue Revascularization using a transferred muscular–vascular pedicle. *Ann. Plast. Surg.* 17, 299–305.
13. Hyakusoku, H., Okubo, M., Umeda, T. A., and Fumiiri, M. (1987). A prefabricated hair-bearing island flap for lip reconstruction. *Br. J. Plast. Surg.* 40, 37–39.
14. Khouri, R. K., Tark, K. C., and Shaw, W. W. (1992). Prefabrication of flaps using an arteriovenous bundle and angiogenesis factors. *Surg. Forum* pp. 597–599.
15. Dickerson, R. C., and Duthie, R. B. (1963). The diversion of arterial blood flow to bone. *J. Bone J. Surg. Am. Vol.* 45A, 356.
16. Woodhouse, C. F. (1963). The transplantation of patent arteries into bone. *J. Int. Coll. Surg.* 39, 437.
17. Orticochea, M. (1971). A new method for total reconstruction on the nose: The ears of donor areas. *Br. J. Plast. Surg.* 24, 225.
18. Albrektsson, T., Branemark, P. A., Erikson, A., and Lindstrom, J. (1978). *Scand. J. Plast. Reconstr. Surg.* 12, 215–223.
19. Fisher, J., and Yang, W. Y. (1988). Experimental tissue molding for soft tissue reconstruction: A preliminary report. *Plast. Reconstr. Surg.* 82, 857–864.
20. Khouri, R. K., Koudsi, B., and Reddi, H. (1991). Tissue transformation into bone in vivo. *J. Am. Med. Assoc.* 266, 1953–1955.
21. Patrick, C. W., Jr., Chauvin, P. B., *et al.* (1999). Preadipocyte seeded PLGA scaffolds for adipose tissue engineering. *Tissue Eng.* 5, 139–151.
22. Patrick, C. W., Jr., Chauvin, P. B., *et al.* (1998). Tissue engineered adipose. *In* "Frontiers in Tissue Engineering" (C. W., Patrick, Jr., A. G. Mikos and L. V. McIntire, eds.), pp. 369–382. Elsevier, Oxford, UK.
23. Lee, K. Y., Halberstadt, C. R., *et al.* (2000). Breast reconstruction. *In* "Principles of Tissue Engineering" (R. P. Lanza, R. Langer and J. Vacanti, eds.), pp. 409–423. Academic Press, San Diego, CA.
24. Katz, A. J., Llull, R., *et al.* (1999). Emerging approaches to the tissue engineering of fat. *Clin. Plast. Surg.* 26, 587–603.
25. Cao, Y. L., Lach, E., *et al.* (1998). Tissue engineered nipple reconstruction. *Plast. Reconstr. Surg.* 102, 2293–2298.

26. Billings, E., Jr., and May, J. W., Jr. (1989). Historical review and present status of free fat graft autotransplantation in plastic and reconstructive surgery. *Plast. Reconstr. Surg.* 83, 368–381.
27. Ersek, R. A. (1991). Transplantation of purified autologous fat: A 3-year follow-up disappointing. *Plast. Reconstr. Surg.* 87, 219–227.
28. Mandrup, S., and Lane, D. (1997). Regulating adipogenesis. *J. Biol. Chem.* 272, 5367–5370.
29. Loftus, T. M., and Lane, M. D. (1997). Modulating the transcriptional control of adipogenesis. *Curr. Opin. Genet. Dev.* 7, 603–608.
30. Entemann, G., and Hauner, H. (1996). Relationship between replication and differentiation in cultured human adipocyte precursor cells. *Am. J. Physiol.* 270, C1011–C1016.
31. Novakofski, J. E. (1987). Primary cell culture of adipose tissue. *In* "Biology of the Adipocyte: Research Approaches" (G. J. Hausman and R. J. Martin, eds.), pp. 160–197. Van Nostrand-Reinhold, New York.
32. Strutt, B., Khalil, W., *et al.* (1996). Growth and differentiation of human adipose stromal cells in culture. *In* "Methods in Molecular Medicine: Human Cell Culture Protocols" (G. E. Jones, ed.), pp. 41–51. Humanaa Press, Totowa, NJ.
33. Hausman, G. J., and Richardson, R. L. (1998). Newly recruited and pre-existing preadipocytes in cultures of porcine stromal–vascular cells: Morphology, expression of extracellular matrix components, and lip accretion. *Anim. Sci.* 76, 48–60.
34. Shillabeer, G. Z., Li, Z.-H., *et al.* (1996). A novel method for studying preadipocyte differentiation in vitro. *Int. J. Obes.* 20(3), S77–S83.
35. Van, R. L. R., and Roncari, D. A. K. (1977). Isolation of fat cell precursors from adult rat adipose tissue. *Cell Tissue Res.* 181, 197–203.
36. Dijan, P., Roncari, D. A. K., *et al.* (1983). Influence of anatomic site and age on the replication and differentiation of rate adipocyte precursors in culture. *J. Clin. Invest.* 72, 1200–1208.
37. Kirkland, J. L., Hollenberg, *et al.* (1996). Effects of fat depot site on differentiation-dependent gene expression in rate preadipocytes. *Int. J. Obes. Relat. Metab. Disord.* 20(Suppl 3), S102–S107.
38. Kirkland, J. L., Hollenberg, *et al.* (1994). Effects of age and anatomic site on preadipocyte number in rat fat depots. *J. Gerontol.* 49, B31–35.
39. Hauner, H., and Entenmann, G. (1991). Regional variation of adipose differentiation in cultured stromal–vascular cells from the abdominal and femoral adipose tissue of obese women. *Int. J. Obes.* 15, 121–126.
40. Crandall, D. L., Hausman, G. J., *et al.* (1997). A review of the microcirculation of adipose tissue: Anatomic, metabolic, and angiogenic perspectives. *Microcirculation* 4, 211–232.
41. Silverman, K. J., Lund, D. P., *et al.* (1988). Angiogenic activity of adipose tissue. *Biochem. Biophys. Res. Commun.* 153, 347–352.
42. Zhang, Q. X., Magovern, C. J., *et al.* (1997). Vascular endothelial growth factor is the major angiogenic factor in omentum: Mechanism of the omentum-mediated angiogenesis. *J. Surg. Res.* 67, 147–154.
43. Varzaneh, F. E., Shillabeer, G., *et al.* (1994). Extracellular matrix components secreted by microvascular endothelia cells stimulate preadipocyte differentiation in vitro. *Metab. Clin. Exp.* 43(7), 906–912.
44. Erol, O. O., and Spira, M. (1990). Reconstructing the breast mound employing a secondary island omental skin flap. *Plast. Reconstr. Surg.* 86, 219–227.
45. Marschall, M. A., Gigas, E. G., *et al.* (1990). The omentum in reconstructive surgery. *In* "Mastery of Plastic & Reconstructive Surgery" (M. Cohn and R. M. Goldwyn, eds.), Vol. 1, pp. 95–101. Little, Brown, Boston.
46. Sugawara, Y., Harii, K., *et al.* (1998). Reconstruction of skull defects with vascularized omentum transfer and split calvarial bone graft: Two case reports. *J. Reconstr. Microsurg.* 13, 101–108.
47. Kral, J. G., and Crandall, D. L. (1999). Development of a human adipocyte synthetic polymer scaffold. *Plast. Reconstr. Surg.* 104, 1732–1738.
48. Marler, J. J., Guha, A., *et al.* (2000). Soft-tissue augmentation with injectable alginate and syngeneic fibroblasts. *Plast. Reconstr. Surg.* 105, 2049–2058.
49. Duranti, F., Salti, G., *et al.* (1998). Injectable hyaluronic acid gel for soft tissue augmentation. *Dermatol. Surg.* 24, 1317–1325.
50. Vacanti, J. P., Atala, A., *et al.* (1998). Breast tissue engineering. U.S. Pat. 5,716,404.
51. Kubo, Y., Kaidzu, S., *et al.* (2000). Organization of extracellular matrix components during differentiation of adipocytes in long-term culture. *In Vitro Cell. Dev. Biol.* 36, 38–44.
52. Kawaguchi, N., Toriyama, K., *et al.* (1998). *De novo* adipogenesis in mice at the site of injection of basement membrane and basic fibroblast growth factor. *Cell Biol.* 95, 1062–1066.
53. Yuksel, E., Weinfeld, B., *et al.* (2000). Increased free fat-graft survival with the long-term, local delivery of insulin, insulin-like growth factor-I, and basic fibroblast growth factor by PlGA/PEG microspheres. *Plast. Reconstr. Surg.* 105, 1712–1720.
54. Yuksel, E., Weinfeld, A. B., *et al.* (2000). *De novo* adipose tissue generation through long-term, local delivery of insulin and insulin-like growth factor-1 by PLGA/PEG microspheres in an *in vivo* rat model: A novel concept and capability. *Plast. Reconstr. Surg.* 105, 1721–1729.
55. Serrero, G. (1987). EGF inhibits the indifference of adipocyte precursors in primary cultures. *Biochem. Biophys. Res. Commun.* 146, 194–202.
56. Kras, K. M., Hausman, D. B. *et al.* (2000). Tumor necrosis factor stimulates cell proliferation in adipose tissue-derived stromal–vascular cell culture: Promotion of adipose tissue expansion by paracrine growth factors. *Obes. Res* 8, 186–193.